DISSERTATION

EVALUATION OF ALLOGENEIC FREEZE-DRIED PLATELET LYSATE AND CONDITIONED SERUM IN JOINT TISSUES UNDER INFLAMMATORY CONDITION IN VITRO

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Livia Camargo Garbin

Department of Clinical Sciences

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Doctoral Committee:

Advisor: David D. Frisbie

C. Wayne McIlwraith
John D. Kisiday
Christine S. Olver

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ABSTRACT

EVALUATION OF ALLOGENEIC FREEZE-DRIED PLATELET LYSATE AND CONDITIONED SERUM IN JOINT TISSUES UNDER INFLAMMATORY CONDITION IN VITRO

Several biological therapies have been developed with the objective of modulating complex inflammatory pathways present in various degenerative diseases such as osteoarthritis (OA). Two examples of biological therapies are platelet-rich plasma (PRP) and autologous conditioned serum (ACS). Both therapies showed potentially disease modifying properties in different tissues, including cartilage. PRP is a blood derived product rich in growth factors that demonstrated anti-inflammatory properties and to promote healing in patients suffering from OA. ACS is also a blood-derived product that has been shown to provide a favorable ratio of interleukin-1 antagonist receptor protein (IL-1Ra) relative to agonist interleukin-1(IL-1β) as well as other growth factors that could inhibit the progression of OA. ACS has shown symptom and disease modifying properties in experimental equine OA.

Although positive effects have been seen with the use of both products, practical issues in preparation and storage limit the clinical use. For instance, the variability between patients, protocols of preparation for PRP and ACS and application interfere with the efficacy of the therapy. Additionally, the long process for production of such therapies, particularly for ACS, preclude its immediate use. Finally, these products must be stored at low temperatures (least at -20°C) to preserve its properties which is not possible in many small clinics or in the field.

Therefore, the main objective of this study is to compare the effects of an allogeneic freeze-dried version of a platelet-derived product and conditioned serum (CS) to the current standard of therapy in cartilage and synovium under inflammatory conditions in vitro. An allogeneic freeze-dried version of these therapies would provide a more homogeneous, stable and user-friendly product for clinical use. In this study, PRP was lysed through freeze-thawing and therefore platelet lysate (PL) was the platelet derived product used in this study. We compared the allogeneic freeze-dried PL and CS to the autologous fresh/frozen preparations, which were comparable to the what is used in the field currently.

Three experiments were performed to evaluate the effects of allogeneic freeze-dried PL and CS; the first one, tested the effects of different formulations of PL in cartilage. In this experiment, PL was tested in three different treatments (frozen, freeze-dried and filtered freeze-dried), three different concentrations (1.5x, 3x, and 6x more concentrated in platelets compared to whole blood baseline) and in autologous or allogeneic conditions. All those different formulations were tested in cartilage under inflammatory conditions in vitro. For the other two experiments performed in this study, different formulations of CS were tested. CS was tested in three different treatments (frozen, freeze-dried and filtered freeze-dried), two different concentrations (10% and 30% volume/volume diluted in media) and in autologous and allogeneic conditions. In one CS experiment, different formulations of CS were tested in cartilage, while the other CS experiment tested the different formulations of CS in synovium. Both CS experiments in vitro were under inflammatory conditions. The main effects evaluated in this study for all the three experiments were; treatment, concentration and allogenicity (allogeneic and autologous CS or PL).

For each experiment described, eight skeletally mature (2-5 years) and health horses were used for blood and tissue harvesting. Equine blood was harvested for the preparations of the different formulations of PL or CS. Cartilage or synovium were collected from these same horses after euthanasia (for reasons non-related to this experiment). The explants were cultured ex-vivo for 10 days total. In cartilage explants, sixteen hours before treatment the explants were labeled using $^{35}SO_4$ isotope. On day 0 and 4 of the experiment, the explants were stimulated with 10 ng/mL of IL-1β to induce a pro-inflammatory environment and exposed to different formulations of PL or CS. To evaluate the protective effects of PL or CS in cartilage, total glycosaminoglycan (GAG) in cartilage explants and media were measured using dimethyl-methylene blue (DMMB) as a measure of cartilage synthesis/degradation. The $^{35}SO_4$-labeled GAG in cartilage explants and in media were evaluated as a measured of newly synthetized GAG degradation using Alcian Blue assay. Finally, to further assess the anti-inflammatory properties of CS and PL, gene expression for IL-1β, COX-2, ADAMTS-4, ADAMTS-5 and MMP-1 were evaluated for cartilage, and COX-2 and IL-1β were evaluated for synovium.

The total GAG and $^{35}SO_4$-labeled GAG in cartilage and media samples treated with different formulations of either PL or CS were not statistically different compared to the controls. Therefore, the formulations of PL and CS used in this study failed in protecting cartilage and synovium from inflammatory stimulus in the model implemented. Such effects might be related to cytokine content of PL and CS obtained in this study, which was not able to protect cartilage from the catabolic stimulation induced by IL-1β. Additionally, in PL treated samples, this treatment induced upregulation of all the pro-inflammatory genes evaluated in cartilage, suggesting potential pro-inflammatory effect. Such findings were somewhat surprising to the authors once the gene expression obtained in cartilage treated with PL did not reflect the

changes observed in cartilage matrix (i.e.PL did not present increase in catabolism of the cartilage matrix). Thus, it's possible that PL components could have interfered with other factors not at the gene expression level, or with other pathways that were not investigated in this study, which could justify these results. In CS experiment, the use of this product only induced upregulation of MMP-1 in cartilage and IL-1β in synovium. For all the other genes evaluated the use of CS did not present significant effect.

Frozen and freeze-dried preparations seemed to regulate gene expression differently in cartilage samples treated with PL. Samples treated with frozen PL presented significantly more upregulation of ADAMTS-5 (about 11-fold higher), compared to freeze-dried treated samples. IL-1 β was significantly more upregulated for frozen autologous PL treated samples (about 90-fold higher) compared to freeze-dried allogeneic PL treated samples. MMP-1 was significantly more upregulated for freeze-dried PL treated samples compared to frozen (about 2-fold) and finally, freeze-dried allogeneic PL treated samples had significantly more upregulation of COX-2 (about 2-fold) compared to frozen allogeneic PL group. Such differences though, did not reflect in significant changes in matrix at the end of the experiment. Regarding CS, gene expression of the pro-inflammatory genes evaluated were not significantly different between the different formulations of CS used in either cartilage or synovium, but differed depending on the tissue. In cartilage, CS did not present a significant effect in COX-2 and IL-1β expression however in synovium it did upregulated levels of IL-1β, significantly (about 7-fold).

The PL effects in cartilage matrix were concentration dependent, and intermediate platelet concentrations showed better outcomes in this study. With CS, concentration did not demonstrate to have a significant effect in the model used. Autologous and allogeneic condition had significant effects in cartilage gene expression for samples treated with PL, but not in

cartilage matrix. For CS on the other hand, allogenicity did not demonstrate to have a consistent significant effect during the experiment or in cartilage or synovium explants at the termination of the study, and were considered overall equivalent.

In conclusion, the proposed allogeneic freeze-dried PL in this study demonstrated to modulate inflammation differently compared to the current state of practice used although such effects did not interfere with cartilage matrix metabolism. With CS, the allogeneic freeze-dried version of the product proposed in this study was equivalent to the current standard of practice used in the field and could offer a more homogeneous and user-friendly product for use. The investigation of the allogeneic freeze-dried version of PL and CS in in vivo models to evaluate the modulatory effects of these product in joints is necessary to confirm these results.

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PREFACE

Osteoarthritis (OA) is a complex disease resulting from the increase in catabolic activity in oppose to anabolic activity, in joint tissues leading to the progressive degeneration of the joint. OA affects millions of people and animals decreasing substantially productivity and quality of life. To relief such burden, a series of treatments have been developed, from pharmacological therapies and dietary supplementation to surgery. Once OA results from the interaction of several inflammatory pathways, the potential modulation of such pathways could alleviate symptoms and diminish or even cease the progression of the disease. Thus, a series of biological treatments have been developed to ameliorate healing of musculoskeletal diseases, including OA. Such therapies provide a wide range of bioactive factors that modulate inflammatory response and stimulate cellular recruitment, growth and morphogenesis. Specifically, platelet-rich plasma (PRP) and autologous conditioned serum (ACS) have been widely adopted to augment healing of tissues with low healing potential such as cartilage, with frequent success.

However, the use of such therapies has its limitations. One limitation it's the variability in protocols for production, application and activation (PRP) as well as the patient's own blood characteristics. This results in a product with inconsistent content of biological factors, leading to contradictory outcomes. Another limitation it's the laborious and time consuming protocols to produce such therapies. Such protocols do not allow product's availability for immediate use, which is a potential issue specially for clinicians in the field. One additional limitation is the need for storage at low temperatures (at least -20°C) to preserve the product's properties, which limits the use of biological therapies in many clinics and in the field.

Therefore, the present study proposed a creation of an Allogeneic Freeze-dried version of Platelet lysate (PL- previously lysed PRP) and Conditioned Serum (CS) in horses. Such protocol could create a product with more homogeneous cytokine profile, possibly resulting in more consistent results for this therapy. Moreover, once this product would be created using blood from donor horses, it would allow a more widespread use. In addition, the freeze-dried form creates a stable product that can be stored at room temperature. Therefore, this version would allow the immediate use of a more consistent product that does not require any equipment for its preparation or storage. Additionally, this product could be re-suspended for immediate application into the joint, which would provide a much more user-friendly version of biological therapy for use in the field. Therefore, in this study three hypotheses were tested; 1) Freeze-dried version of the proposed products (PL and CS) will have similar effects compared to frozen preparations in cartilage and synovium stimulated with IL-1β, 2) Allogeneic version of the proposed products (PL and CS) will have similar effects compared to autologous preparations in cartilage and synovium stimulated with IL-1β, and 3) Effects of PL and CS in cartilage and synovium will be concentration dependent.

To address the hypotheses, 3 experiments were performed. One experiment explored the use of different formulations of PL in cartilage under inflammatory conditions. The other two, explored the use of different formulation of CS in cartilage and synovium under inflammatory conditions. In each experiment, 8 horses were utilized for blood harvest for the preparation of the different PL and CS treatments (frozen, freeze-dried and filtered freeze-dried). Then cartilage or synovium explants were harvested from the same horse as the blood samples were collected and submitted to inflammatory conditions in vitro for 10 days. Samples were exposed to PL or CS different treatments, under autologous or allogeneic conditions and in different concentrations.

Media and cartilage explant samples were collected after the 10 days of culture and submitted to biochemical analysis to evaluate the effects of the different PL and CS formulations in cartilage matrix. Cartilage and synovium explants were submitted gene expression analysis, to verify the modulatory effects of the different formulations of PL and CS. Data was evaluated using a mixed model analysis of variance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Use of biological therapies for osteoarthritis

Osteoarthritis (OA) is a disease that negatively affects multiple tissues within the joint such as cartilage, synovial membrane and subchondral bone. This common form of arthritis tends to progress over time resulting in cartilage degeneration[1]. This disease was estimated to affect over 27 million people in U.S., being considered a main cause of disability in adults[2]. Despite its importance, there is no current effective cure for OA. Therefore, the main objective of OA therapy is to control pain and improve joint function.

In OA, inflammation is associated with symptoms and progression of the disease[3]. Consequently, non-steroidal anti-inflammatory (NSAIDS) [4] and analgesic drugs as well as intra-articular corticosteroids and visco-supplementation[5] are common medications used to manage OA. In many instances, the lack of long-term effectiveness results in progressive degeneration of the joint which manifests as OA. For this reason, new approaches that could potentially inhibit or modulate the progression of inflammation are warranted. Such therapies could result in more promising clinical and functional outcomes for patients. Biological therapies such as platelet-rich plasma (PRP)[6] and autologous conditioned serum (ACS)[7] offer hope for clinicians and researchers as new disease-modifying approach for OA. These products are derived from the patient's own blood and are rich in cytokines and growth factors that modulate inflammation and enhance tissue healing. Clinically, PRP and ACS have been widely viewed as means of augment healing of tissues with low healing potential such as articular cartilage[6] [7]. Although proof-of-principle in vitro has been stablished for biological therapies, limitations preclude its widespread use. One of the main limitations is the variability in the final product used [8, 9], which leads to

potentially different outcomes. These limitations could be explained by the lack of standardization in the protocols for production, application of biological treatments as well as patient's variability. Thus, the objective of this review is to cover important aspects related to the use of PRP and ACS as biological therapies for OA and its limitations.

1. OA disease

OA is a degenerative disorder of movable joints characterized by cell stress and extracellular matrix degeneration initiated by micro and macro-injury. Such insult triggers a maladaptive response resulted from abnormal tissue metabolism leading to anatomic and physiologic imbalance. This metabolic disparity leads to cartilage loss, bone remodeling, osteophyte formation, and joint inflammation that culminates in loss of function[10, 11]. Because a variety of tissues within the joint may be affected, OA is considered a disease of the joint organ which may lead to joint failure[12].

1.1 OA in the horse

Spontaneous OA is a common cause of lameness observed in horses in which age of the animal may vary[13], and is responsible for up to 60 % of the lameness cases[14, 15]. In fact, including indirect costs, the financial impact of lameness in the horse population can reach up to $ 1 billion per year in the United States [16].

In the horse, the metacarpophalangeal joint is the most commonly affected by spontaneous OA, followed by the carpal joints. OA in these joints develop linear erosions, wear lines in cartilage and osteochondral fragments[17, 18]. As in humans, OA in horses can be potentially initiated through the inflammatory response of tissues in the joint other than cartilage, such as the synovial membrane[19]. Consequently, the inflammatory response triggered might

lead to the progression of OA, bringing serious financial burden and concerns related to animal's welfare.

1.2 OA in humans

OA is one of the main causes of disability in aging population[11]. OA and affects nearly 12.1% of seniors in the United States, resulting in an annual cost of 89.1 billion, which is largely attributed to work-related losses and home-care costs [11] . This disease is one of the main causes of hip and knee replacement[20] [10]. Although the age-standardized prevalence of hip and knee OA have not shown much change in twenty years (1990-2010), the number of people with disabilities related to OA increased from 10.5 to 17.1 million[10]. Thus , in human OA causes an enormous burden and therefore development of effective ways to modulate inflammation in OA and possibly reduce or stop the progression of this disease are needed.

2. Pathogenesis of OA

OA pathogenesis involves multiple etiologic factors including mechanical and biochemical stimulation, inflammation and genetic abnormalities. These factors contribute to the imbalance in the homeostasis of the joint leading to cartilage destruction. From the mentioned factors, the focus of this review is on contributions from inflammation to OA.

Inflammation can initiate and propagate OA. Cytokines such as IL-1β and TNF-α are well known for their paramount role in the initiation and progression of OA. Other cytokines and pro-inflammatory enzymes may perpetuate inflammation, while anti-inflammatory cytokines can modulate or inhibit this process[21]. Next, important aspects related to inflammation in OA are reviewed.

2.1 Inflammation and relevant cytokines in OA

Several studies demonstrated the impact of inflammation in OA progression and symptoms[22]. In joint disease, the main sources of cytokines and chemokines triggered by IL-1β and TNF- α[23] are chondrocytes, synovial macrophages and fibroblasts[24, 25] . Inflammatory cells such as macrophages actively participate in OA disease and are paramount for its progression [26]. The presence of these cells are believed to play an important role in stimulating and inducing release of interleukin 1 β (IL-1β) and tumor necrosis factor- α (TNF-α) in synoviocytes[25]. Those cytokines are among the most important catabolic regulators in OA[27].

In homeostatic conditions, anabolic and catabolic agents are balanced in cartilage. In osteoarthritis thought, there is an imbalance between anabolic and catabolic factors leading to cartilage matrix degradation, which is triggered by the pro-inflammatory cytokines such as IL-1β and TNF-α[1] [28]. Once inflammation is initiated (i.e. through trauma), the downstream activity of IL-1β and TNF-α triggers the stimulation and increase of a series of pro-inflammatory cytokines and chemokines (such as IL-8) resulting in the release of degradative enzymes, leading to matrix degradation (Figure 1)[23, 29-31] .In cartilage, the most important enzymes are metalloproteinases (such as MMP-1, MMP-3, MMP-8 and MMP-13) and aggrecanases (such as A desintegrin and metalloproteinase with thrombospondin motifs 4 and 5 [ADAMTS 4 and 5][3], leading to the destruction of important matrix components, such as collagen type II and proteoglycans[12] respectively (Figure 1.1). Besides the increase of catabolic factors, TNF-α and IL-1β decline the synthesis of antioxidants such as superoxide dismutase, catalase and glutathione peroxidase [32], that counter-balance the degradative effects of reactive oxygen species (ROS), culminating with an increase in cartilage degradation.

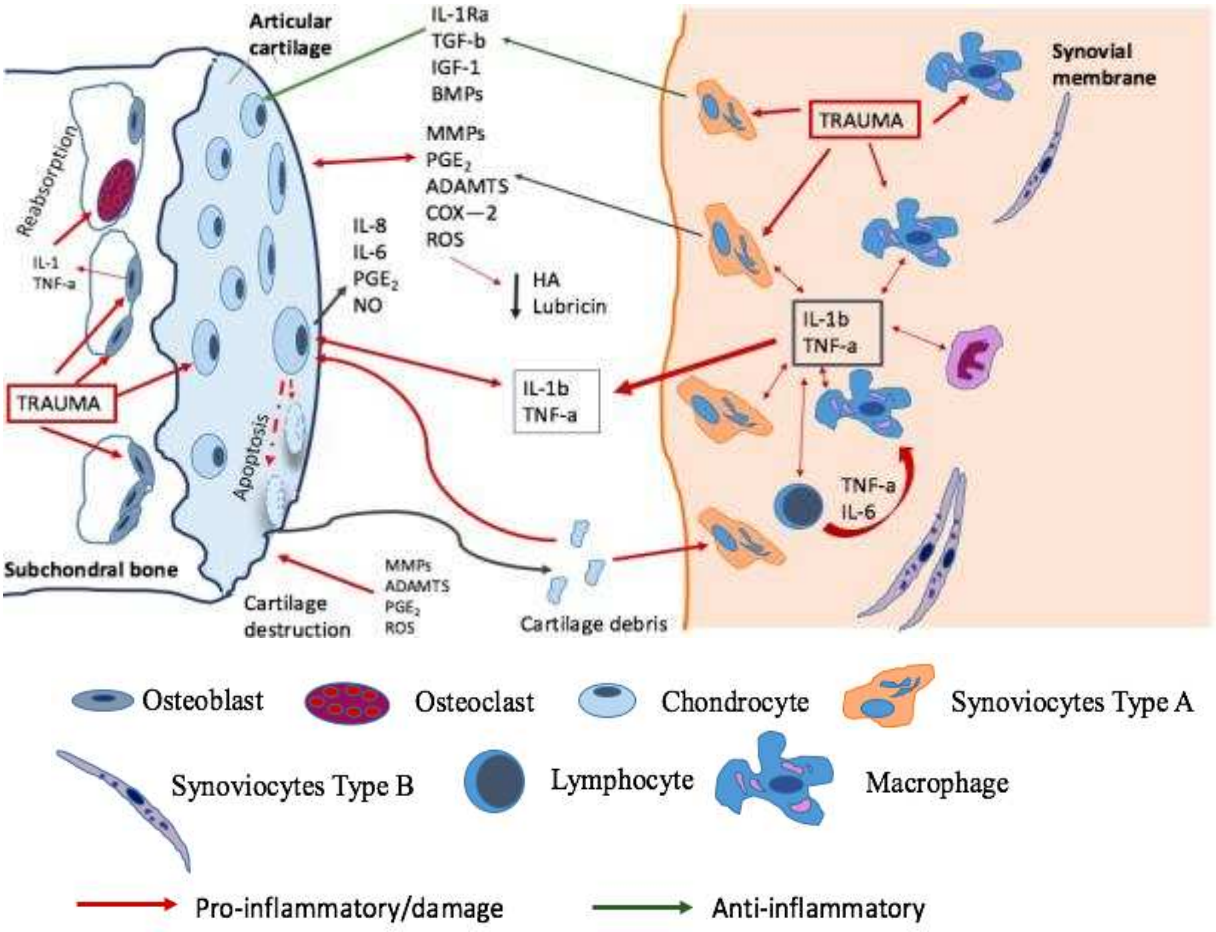

Figure 1.1: Diagram of main cytokines and cells involved in inflammation during osteoarthritis. Green arrows indicate the upregulation of anti-inflammatory factors; red arrows indicate upregulation of pro-inflammatory factors; dotted red arrows indicates apoptosis. IL-Ra= interleukin-1 receptor antagonist protein, TGF-B= transforming growth-factor beta, IGF-1=insulin-like growth factor, BMPs= bone morphogenetic proteins, MMPs=metalloproteinases, PGE_2= prostaglandin E_2, ADATMS= aggrecanases, COX-2= cyclooxygenase 2, ROS= reactive oxygen species, NO= nitric oxide, IL-8= interleukin-8, IL-6= interleukin-6, IL-1β= interleukin 1 beta, TNF-α= tumor necrosis factor alfa. Diagram based on the references: Kapoor et al (2011)[1], Lieberthal, Sambamurthy and Scanzello, 2015[3] and McIlwraith, 2005[33].

As IL-1β and TNF-α, other cytokines such as IL-17, IL-18 and leukemia inhibitory factor (LIF) also play a catabolic role in OA, leading to cartilage degradation. Some factors such as IL-16 and IL-8 on the other hand, have dual roles and can either induce synthesis or matrix degradation [21]. Finally, as a regulatory mechanism in OA, anti-inflammatory cytokines such as

IL-4, IL-10 and interleukin receptor antagonist protein (IL-1Ra) can reduce or inhibit the action of pro-inflammatory cytokines[21]. The downregulation of pro-inflammatory pathways reduce the expression of paramount enzymes and cytokines that propagates OA [34, 35].Besides growth factors such as insulin-like growth factor (IGF), transforming growth factor (TGF)-β, bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) are considered anabolic for cartilage, opposing the degradative action of destructive cytokines and enzymes [21]. These anabolic factors are naturally found in OA joints and are important to modulate and counteract inflammation.

For this reason, anti-cytokine therapies that control the downstream effects of IL-1β and TNF-α and use of growth factors that promote matrix synthesis are desirable alternatives for treatment of OA[1]. These new biochemical modulatory therapies aimed to restore the anabolic-catabolic balance within the joint and modulate the progression of OA, reducing pain associated with it[36, 37].

3. Therapies for OA

The current options for the treatment of OA include nonpharmacological, pharmacological and surgical approaches[38]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are considered to be efficient in providing analgesia and for that reason those are the most commonly recommended drugs to be used for the relief of pain caused by inflammation[4]. Yet, NSAIDs side effects such as gastrointestinal, cardiovascular and renal signs, has sustained interest in developing alternative approaches[4]. Intra-articular injections of corticosteroids and hyaluronic acid (HA) are good alternatives for managing OA[4].

Specifically, corticosteroids can reduce the action of inflammatory mediators such as IL-1β, TNF-α and cycloxigenase-2 (COX-2)[39]. Still, the lack of long-term effect of such therapies lead to the need of new treatments that could modulate and stop the progression of OA.

Based on the action of molecules found naturally in OA joints, some anti-inflammatory cytokines as well as the blockage of pro-inflammatory cytokines (i.e. with the use of antibodies for example) have been used as therapies for OA. For instance, antibodies against IL-1β and TNF-α as well as inducible nitric oxide (iNOS) inhibitors have been developed (reviewed in [22]). Such treatments have been demonstrated to be associated with the decrease of inflammatory mediators in OA[40]. Drugs with the objective of blocking or antagonizing the effects of TNF-α (such as infliximab, etanercept and adalimumab)[41], and IL-1β (such as Anakira®)[7] demonstrated clinical efficiency. Therapies including the use of interleukins as IL-4, IL-10 and IL-13 that inhibits the effects of IL-1β[42] as well as inhibition of degradative enzymes such as metalloproteinases and aggrecanases[43, 44] have also been considered. Nonetheless, the studies involving these medications for the treatment of OA had small number of patients and short duration and therefore, clinical efficacy for the widespread use of such therapies has yet to be stablished.

Biological therapies have gained attention given their ability to provide beneficial bioactive factors that can modulate inflammation. Various factors are found in blood and can be enhanced through proper blood processing. Numerous studies have explored the potential of blood-derived products such as platelet-rich plasma (PRP) and autologous conditioned serum (ACS) to enhance healing and modulate inflammatory processes, including OA, with encouraging results [45, 46] . PRP and ACS are two of the most notable and are explored with more detail as alternative therapies for OA in the following text.

3.1 Platelet-rich products and its application in OA

Several studies have evaluated and demonstrated the beneficial biological effects of platelet-rich products in musculoskeletal disease using in vitro and in vivo experiments [47-49]. Due to the growth factors released by the alpha-granules and their capacity to modulate tissue healing and inflammation, platelet-derived products have called attention of researchers as a potential treatment for osteoarthritis[48, 50]. Yet, due to the lack of standardization in protocols for PRP production[51] [52], platelet activation[53] and treatment application, great variability in outcome parameters is observed with the use of platelet-derived products. Thus, although the proof-of-principle of platelet-derived products has been demonstrated in vitro, the evidence-based efficiency for its clinical application remains to be elucidated.

Subsequently, the purpose of this review is to clarify aspects of platelet physiology and activation that are relevant to platelet-rich plasma therapy, as well as the importance of growth factors in osteoarthritis and the use of platelet-rich plasma as a therapy for this disease.

3.1.1 Functions of platelets

In platelets, proteins are stored in dense granules, alpha granules and lysosomes . These proteins are essential for the formation of thrombus and hemostasis, chemotaxis of leukocytes as well as tissue healing[50]. Platelets contain over 800 proteins that with modification in their structure results in more than 1500 bioactive factors[54]. The dense granules (or γ granules) contain small molecules such as adenosine di-phosphate (ADP), tri-phosphate (ATP) and serotonin that promote hemostasis at the site of vascular damage[55]. The lysosomes release hydrolytic enzymes that are believed to be important for thrombus remodeling[56]. The α-granules are the most abundant granules in the platelet[57]. These granules contain the growth factors that are chemotactic to cells, and stimulate cell migration, proliferation and matrix synthesis[58].

Growth factors found in α-granules include PDGF, FGF and TGF-β among others. Besides the growth factors, the α-granules contain a great amount of further essential molecules such as hemostatic factors , proteases and necrosis factors. [59].

The growth factors present within platelets not only play an important role in inflammation but also in healing and for this reason, those have been used therapeutically to stimulate tissue repair[60]. When used therapeutically, growth factors can be applied directly into the site of the lesion or in synergy with other growth factors, with the use of platelet derived products.

3.1.2 Platelet-derived products

Platelet-rich plasma (PRP) has been described in the literature essentially as a volume of plasma containing an increased concentration of platelets relative to whole blood[61] [62]. This product is prepared based on the centrifugation of peripheral whole blood, resulting in a high concentrated platelet sample[63]. The first report of PRP use was in the 1970s[63]. In the following years, greater attention was given to gravitation forces and times of centrifugation, which was noted to influence the composition of the PRP. In the 1990's, the use of PRP was further proposed as a potential new bioscaffold [61]. Based on the different preparation methods, PRP has diversified into a variety of products according to their cellular or fibrin content [61]. PRP can be delivered in multiple forms, through local injections or infiltrations, and with other biomaterials, scaffolds or cells [64] [65]. PRP is the subject of many papers exploring its effect in the past 10 years (Figure 1.2). Specifically, hundreds of papers were published investigating the therapeutic potential of PRP in musculoskeletal disease (Figure 1.3).

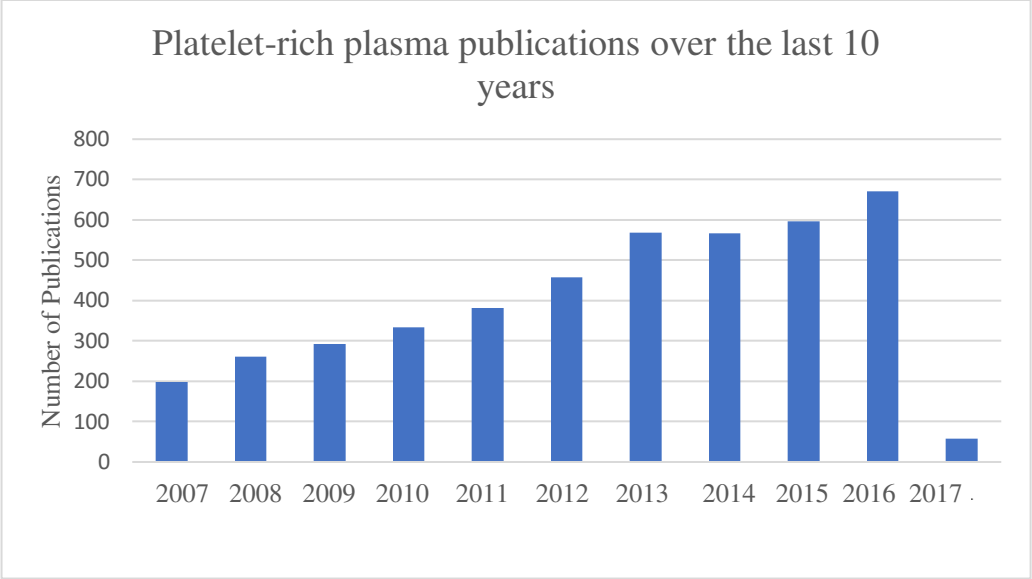

Figure 1.2: Platelet-rich plasma publications over the last 10 years: Number of publications in platelet-rich plasma per year over the last 10 years (source: PubMed (January 2017). Search string: "platelet-rich plasma"). The number of publications increased over the years and achieved a "plateau" between 2013 and 2015, continuing the increase in 2016.

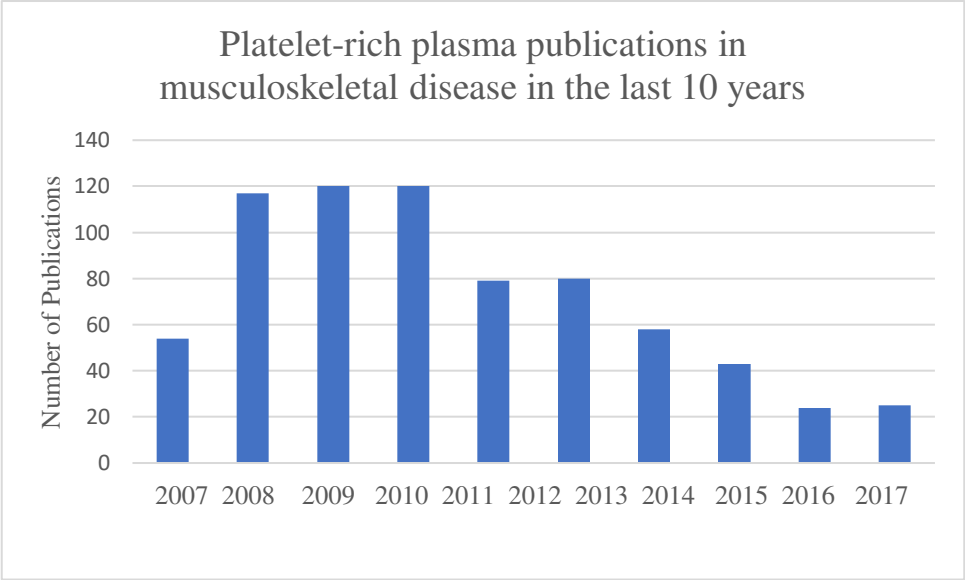

Figure 1.3: Platelet-rich plasma publications in musculoskeletal disease over the last 10 years. Number of publications in platelet-rich plasma for musculoskeletal disease treatment per year over the last 10 years (source: PubMed (January 2017). Search string: platelet-rich plasma in musculoskeletal disease). The number of publications increase drastically from 2007 to 2008, achieving a peak in 2009/2010 and decreasing until 2016.

PRP contains growth factors that can potentially optimize tissue healing such as; TGF-β, PDGF, IGF and VEGF[60]. Most growth factors are released within 1 hour of platelet activation and their half-life usually ranges from minutes to hours[60]. Specifically, TGF-β is the most abundant growth factor contained in PRP [62, 66] and has been demonstrated to decrease catabolic activities induced by IL-1β and TNF-α in chondrocytes [62, 66]. Likewise, IGF has been shown to increase proteoglycan synthesis in chondrocytes [67]. Because of the anabolic and modulatory effects of growth factors, PRP has been used in several musculoskeletal tissues restore extracellular matrix restoration properties in ligaments[68], tendons[69] and bone [70]. Such positive results are encouraging and indicate that PRP could be a reliable alternative for disease and symptom modifying therapies in musculoskeletal disease.

3.1.3 Platelet-derived products in osteoarthritis

For OA, the positive effects obtained with the use of PRP is believed to be explained by the milieu of growth factors offered by this product. Several studies demonstrated the positive modulatory effects of PRP by reducing multiple catabolic effects of IL-1β [62]. For instance, in an osteoarthritis co-culture in vitro model, the anti-inflammatory properties of PRP were characterized [47]. Two different preparations of PRP (leukocyte-rich and leukocyte poor PRP), reduced the expression of pro-inflammatory markers and increase the expression of anti-inflammatory markers at 24, 48 and 72 hours in a co-culture study [47]. Moreover, Cerza et al (2012) [71] treated patients with different levels of knee OA with PRP and hyaluronic acid (HA). The authors observed that patients achieved lower Western Ontario and McMaster Universities Arthritis score (WOMAC) when treated with PRP in comparison with HA, particularly in patients with grade III OA. However, the study was not blinded or placebo-controlled and therefore the positive results obtained with PRP use should be considered with cautious.

The use of PRP shown beneficial effects in joints affected by OA in clinical trials. In Kon et al (2011) [49], 150 patients with different degrees of OA were either treated with 1 injection of hyaluronic acid (HA) in different molecular weights, or 3 weekly injections of PRP. The use of PRP showed significant symptomatic and functional improvement up to 6 months after treatment, being superior when compared to HA [49]. In another clinical trial, 109 patients with OA were treated with 3 weekly injections of either PRP or HA. PRP injections offered a significant subjective clinical improvement in younger individuals with knee osteoarthritis treated with PRP in the study. However, however for middle-aged patients with moderate signs of OA PRP results were not better compared to HA [72]. These results suggest that PRP requires further investigation and optimization to be used efficiently in OA joints.

Some authors believe that the positive results of PRP are mainly related to symptom modifying effects rather than disease modifying properties [72]. This could be related to the influence that platelets would have on joint homeostasis and modulation of cytokine levels which could justify the temporarily clinical improvements, but not necessarily interfering with the degenerative progression in the cartilage [73]. On the other hand, clinical improvements were still observed in a 2-year clinical trial, mainly in younger patients treated with PRP [72]. Thus, the outcomes resultant from PRP use are variable between studies and it is still not completely understood how PRP components regulate inflammation within the joint.

The various outcomes presented here with the use of PRP might be explained by a wide range of factors that influence PRP efficacy. Such factors are further presented.

3.1.4 Factors that influence PRP effects

The effects of PRP in different tissues may be affected by several factors, such as cellular PRP composition, patient's blood variability, and protocols for platelet activation and PRP application.

Regarding the cellular content of PRP, earlier studies have shown that lower concentrations of platelets induce an insufficient or sub-optimal cell stimulation, whereas higher concentrations can promote apoptosis [74] [75], impairing tissue healing. Such results might be explained by a platelet/growth factor concentration negative feedback. After growth factors reach a certain concentration, their receptors are downregulated and desensitized reducing its effect [76, 77] .Thus, the dose-response curve of the tissue to PRP effects is not linear and inhibitory effects on cells take place once a certain concentration of platelets has been reached [75]. Some authors believe that an intermediate concentration of platelets in the PRP (2 to 6 times the concentration in the whole blood) might be more indicate for use [68].

Deleterious effects have been observed in highly concentrated platelet in PRP and this has been suggested as related to a concurrent higher concentration of leukocytes present within the product [78-79], which can depend on the system used to prepare PRP [80, 81]. Leukocytes in PRP are believed by some authors to be deleterious for tissue healing because of the release of ROS, pro-inflammatory cytokines and degradative enzymes[60, 73]. In contrast, other authors believe that leukocytes are an important source of enzyme and cytokines which could be important for the prevention of infection[82, 83]. Further investigation to determine the optimal platelet and leukocyte concentration in PRP and how that may affect the different tissues within the joint during OA is needed.

It is important to consider the growth factor content as well as cellular composition in the PRP may vary, not only relative to patient variation, but also according to the time in which the blood is collected. Significant variation on both cellular and growth factor content of PRP were reported in the same patient when the blood was collected within a period of two weeks[84]. In addition, the growth factor content can vary among patients and is not necessarily correlated with the number of platelets within PRP [62]. Even the composition of the platelet product demonstrated previously to influence in the pattern of growth factors release [85]. In a study done by Schar et al (2015) [85] the authors compared the kinetics of release of growth factors within leukocyte and platelet-rich fibrin, leukocyte and platelet-rich plasma and a natural blood clot in vitro for 28 days. The authors verified that leukocyte and platelet-rich fibrin had higher amount and long-term release of growth factors compared to leukocyte and platelet-rich plasma [85]. Therefore, PRP composition may affect the growth factor content and kinetic of cytokine release which could potentially influence in the anti-inflammatory and healing effects of PRP when applied in tissues.

Additionally, to the PRP cellular composition, the preparation and activation methods used in PRP therapy should be considered as well. The different methods of platelet activation showed to influence in growth factors concentration and the dynamics of their release [84]. Once activated, the platelets release an initial "burst" of growth factors that is followed by a more sustained release. Close to 70% of the growth factors are released within the first 10 minutes of activation and almost 100% of the growth factors content within the platelets are believed to the secreted in the first few hours of activation [65, 86]. Platelets within the PRP products can be activated through endogenous and exogenous methods. The platelets are frequently activated with collagen, ADP, calcium chloride and thrombin, but can also be stimulated with other agents

as chitosan, magnesium and batroxobin [87]. Depending upon the method used, significant changes in cytokine content and kinetic of release can be observed. For instance, collagen has been demonstrated to promote a slower and more sustained released of growth factors over time, while thrombin induced an intense and fast release of platelet contents [88]. Such characteristics in PRP content and cytokine kinetic can affect tissues differently [89], which can be particularly important for organs with multiple tissues such as the joint .

In fact, while some growth factors present in the PRP product are beneficial for some tissues, it can be potentially deleterious to others [60]. For instance, vascular endothelial growth factor (VEGF) promote angiogenesis which is related to increase in tissue healing, however demonstrated negative effects in cartilage healing [60]. Consequently, the study of the properties of the main components and growth factors present within the PRP and its effects in different tissues should be considered for better healing results.

In addition to characteristics of the PRP itself, the condition of the tissue prior to the use of platelet products (acute versus chronic inflammation) and age of the subject submitted to treatment should be considered. Better outcomes in OA joints were reported in younger patients in comparison with older and in more mild cases in comparison with severe ones [90] [62]. It has been postulate that aged patients platelets release fewer growth factors and may to have a lower stimulatory effect on MSCs, compared to younger patients [90].

Finally, the number of treatments in which PRP is applied in patients is another factor that may contribute to the variability in PRP results. Additional treatments with PRP may not be necessarily related to better outcomes. In a double blinded randomized placebo-controlled trial, both 1 and 2 applications of PRP in knees with OA demonstrated similar improvement [73]. On the other hand, in patients with chronic affections multiple injections might be favorable [91]. For

instance, in chronic patellar tendinopathy the application of 3 consecutive PRP injections improved symptoms and function, allowing a fast recovery in athletes [91]. In sum, the protocols applied for PRP might vary according to the tissue and condition in which is implemented.

3.2 Autologous conditioned serum as an alternative for OA treatment

ACS is a blood derived product that is produced following culture of whole blood with medical grade coated beads. This process results in a serum with high concentrations of IL-1Ra, a protein belonging to the IL-1 family. IL-1Ra is produced by many types of cells including chondrocytes, monocytes and synovial fibroblasts during inflammation [92, 93]. This protein competes with IL-1β for occupancy on IL-1 receptors, thereby inhibiting IL-1 activity, being a promising approach for the modulation of OA [94].

3.2.1 Autologous conditioned serum

Since the discovery of IL-1Ra in 1986, many different methods of producing this protein were developed, in order to use it as a potential anti-inflammatory therapy[7]. Arend et al[95] believed that the enhancement of endogenous IL-1Ra may be an efficient therapeutic approach in diseases where IL-1 plays an important role in pathophysiology[95].Consequently, a new method of up-regulating IL-1Ra from whole blood was develop in 2003, and the resulting product was named autologous conditioned serum (ACS, Orthokine®)[96]. Although this product has been slowly validated, it gained fast clinical acceptance in human and veterinary sports medicine, being used in over 60 countries[7, 97]. ACS is produced by incubating the whole blood for 24 hours at 37ºC with medical grade glass beads previously chemically coated beads (such as $CrSO_4$, or cellulose acetate beads, etc)[42, 98]. After incubation, the product is centrifuged and the blood clots and serum is collected and filtrated. This process has been reported to result in a significant increase not only in IL-1Ra levels, but also other anti-inflammatory cytokines and

growth factors, as well as concurrent increase in pro-inflammatory cytokines as IL-1 ß and TNF-α [36, 99].

On the other hand, some studies have demonstrated the concurrent increase in pro-inflammatory cytokines (such as IL-1β) might happen in whole blood incubation [99]. For this reason, the ratio between anti-inflammatory cytokines and pro-inflammatory cytokines (in special IL-1Ra/IL-1) is considered when evaluating the effects of ACS. Different in vivo studies suggest that the concentration of IL-1Ra should be 10 to 100-fold higher than the concentration of IL-1 to block effectively IL-1 receptors and have therapeutic effect [100]. The ratio (IL-1Ra/IL-1ß), seems to be repeatable in different studies and is used as a measure of ACS activity [7] when using this product therapeutically.

The monocytes within the blood are believed to be responsible for the cytokine increase within the ACS. The chemical-cell interaction as well as the surface area of the beads within different ACS kits[46, 99] seems to play a role in the type of cytokine produced by monocytes[7]. Indeed, although the complete protein composition of ACS has not yet been described. However, it has been shown that ACS contains IL-16, IL-4, TNF-α, IL-10, IL-6, fibroblast growth factor b (FGFb), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), IGF-1, platelet-derived growth factor AB (PDGF-AB) and TGF- ß [42, 96].Thus, therapeutic effects of ACS should not only be attributed to IL-1Ra but also to other cytokines within it[7].

3.2.2 Use of ACS as a therapy for OA

Numerous in vivo studies involving different animal models have been conducted to test the efficacy of ACS as a therapy for OA. For instance, in a rabbit meniscectomy model of OA, ACS induced an increase in IL-1Ra concentration in joints that presented up to four weeks after treatment. Reduction of OA progression was also observed in the same study [101].

In a randomized block experiment, surgery induced OA was experimentally induced in horses which were subsequently treated with intra-articular injection of ACS and compared to placebo injections [102]. Animals treated with ACS had decreased pathologic changes in synovial membrane as well as improvement in macroscopic changes in OA affected joints compared to the placebo control group. Therefore, showing disease and symptom-modifying properties. The use of ACS in the equine OA model caused an increase in IL-1Ra on the treated limb but also in the contra-lateral limb as well, although in lower concentrations [102], and the concentrations of IL-1Ra demonstrated to significantly increase over time. Such findings suggest that the intra-articular administration of IL-1Ra might induce an endogenous production of this cytokine the mechanism remains elusive.

Based on these positive results with the use of ACS in the equine OA model, the use of ACS by equine practitioners to treat OA in horses has increased, suggesting acceptance for the use of this product in horses [103].

The use of ACS in human clinical trials has been reported [36, 46]. In a prospective, randomized, multicenter, double-blind, placebo-controlled trial 167 patients received six intra-articular injections of either Orthokin® or placebo [104]. Patients were evaluated for the WOMAC score, the Osteoarthritis Outcome Score (KOOS), knee society rating system and visual analog scale of pain (VAS). The use of ACS did demonstrate improvement in most outcome parameters compared to placebo controlled treated patients however, being significant only for the KOOS score [104].

As well, Rutgers et al (2010) [105] did not observe any effects on cartilage metabolism in OA joints after six injections of ACS compared to unstimulated serum. The authors attributed the limited results to the rapid disappearance of the cytokines from the synovial fluid after injection and suggest that new intraarticular therapies should focus on prolonging presence of the cytokines into the joint space.

Certainly, it is still unknown how long the cytokines injected with ACS intra-articular application are present in the joint [106]. For instance, IL-1Ra has a short half-life of only 4 to 6 hours. In fact, it is uncertain if in vivo the IL-1Ra levels highly correlates with OA symptoms and progression, as in previous study the ratio IL-1Ra/IL-1β in synovial fluid of advance OA patients did not show a correlation with disability and pain [106]. Therefore, it was suggested that in more severe OA cases the blockage of IL-1 receptors might not be enough to abolish cartilage degradation [106]. Indeed, OA is a complex inflammatory process that involves multiple pathways and cytokines that change dynamically during inflammation. For instance, changes in IL-1 receptor sensitivity or expression in cell membranes, along with the presence of soluble receptors of IL-1 (sIL-LRI and sIL-LRII) [107] and other cytokines (as IL-4 and IL-10) [108], interfere directly with IL-1 effects in cells and thus, are likely to play an important function in inflammation within the joints.

Despite the limited studies and the lack of complete understanding of ACS mechanisms, this therapy does provide an autologous option of symptom-modifying treatment of OA. Besides, ACS has shown no adverse effects and showed superior clinical improvement compared to HA in knees with OA [46]. These positive results led this product to be well accepted in the field of sports medicine [7]. Therefore, further the optimization of the protocol of preparation and application for ACS is warranted and may lead to an increase use of this therapy.

4. Limitations of biological therapies in OA

Regardless of the exciting results obtained in PRP and ACS reports, is important to consider these therapies have limitations that precludes their general clinical use as a treatment option for OA.

One of the main challenges related to PRP and ACS use is the variability in its components which are related to the patient, time of collection and the method implemented for its production [46, 99, 109-110]. Particularly for PRP, several commercial kits are available currently for production of these biological therapies, and claim to concentrate the platelets of the whole blood and enhance anti-inflammatory cytokine in serum. For PRP protocols, the main variables are; blood volume used, PRP final volume, cell concentration, number of spins and force (g force or rpm) and method of platelet activation [51]. All those variables lead to essentially different products, making it challenging to compare results between studies.

In PRP research, to facilitate comparison between studies, a standardize classification method (Platelet-Activation-White blood cells –PAW system) has been proposed. The PAW system takes into consideration; the absolute number of platelets, the activation method for the platelets and the presence or absence of white blood cells (and specifically neutrophils) [80]. The implementation of a standardize classification of PRP products would simplify comparisons and interpretation between studies. Indeed, some papers have used this classification method for their PRP, however is still not widely adopted. Similar system has not been developed for ACS classification.

For ACS, there are not as many commercial kits available for its production [96, 99] as there is for PRP. In ACS protocols, the variability is mainly within the cytokine profile obtained [99], which can vary according to the already mentioned factors, but also the kit used [99, 46], time

of incubation [111] and use of additives in the blood (anti-coagulants for instance) [99]. Finally, the protocols for application of both PRP and ACS treatments are especially variable among studies [49,104, 112].

Despite the lack of standardization in protocols and composition of PRP and ACS therapies, further issues preclude its widespread clinical use. These include the need for specific equipment (centrifuges, incubators) and skilled labor to prepare these products as well as the need for storage at low temperatures to preserve its properties. Such equipment and facilities are not available at small clinics or in the field, offering restrictions for clinicians. Those limitations, as well as the time necessary to produce such products restrict the immediate use of these therapies in clinical practice for human and veterinary medicine.

5. Study Goals

To improve the clinical adoption and ease the use of PRP and ACS, this study evaluated allogeneic freeze-dried versions of PRP derived product or conditioned serum (CS) in horses. In this experiment, we used platelet lysate (PL) as our PRP derived product. PL consists in PRP that has been lysed through freeze-thawing.

An allogeneic freeze-dried PL and CS would allow a more homogeneous cytokine profile of the product, potentially leading to more consistent therapeutic results. Besides, if products are created using blood from donor horses (allogeneic) the aliquots could be prepared in advance of the treatment applications, allowing constant availability for use. Additionally, the freeze-drying allows the product to be stable at room temperature, and would not require cold storage to preserve its properties. In fact, freeze-dried platelets showed a shelf life of at least 22 months, with no loss of viability [113, 114] . Hence, the product could be re-suspended for immediate application into the joint. Even in field conditions this product would offer a more user-friendly

option for the use of biologics for treatment of OA. Few previous studies demonstrated that freeze-dried platelet-derived products have similar effect compare to fresh/frozen preparations in culture systems in vitro and in vivo [115, 116]. Moreover, allogeneic PRP demonstrated to enhance bone healing and to not cause any immunogenic issues in a rabbit large bone defect model [70]. Thus, based on this line of thinking and in previous successful reports, this study proposed the following hypotheses for both products:

H1: Freeze-dried version of the proposed products (PL and CS) will have similar effects compared to frozen preparations in cartilage and synovium stimulated with IL-1β.

H2: Allogeneic version of the proposed products (PL and CS) will have similar effects compared to autologous preparations in cartilage and synovium stimulated with IL-1β.

H3: Effects of PL and CS in cartilage and synovium will be concentration dependent.

The hypotheses will be tested by the following aims:

- PL:

Aim 1a. Does freeze-drying PL alters its protective effects in cartilage compared to its frozen preparations? PL treatments (fresh, freeze-dried, filtered-freeze-dried) were used in media at different concentrations (PL:1.5x, 3x and 6x) in the presence of cartilage explants that were stimulated with IL-1ß. In cartilage, sixteen hours prior to treatment $^{35}SO_4$ was added to the medium to be incorporated into the explants. At the end of 10 days of culture, explants and medium were collected for analysis. At the termination of the experiment, total glycosaminoglycan (GAG) in media and cartilage and $^{35}SO_4$ labeled GAG left in cartilage matrix were quantified using dimethyl-methylene blue (DMMB) assay and Alcian blue respectively, as indicators of matrix degradation. In addition, mRNA expression of pro-inflammatory cytokines was used to evaluate the modulatory effects in inflammation for cartilage.

Aim 2a. Can allogeneic PL be used in place of autologous? Half of the PL were pooled together to create the allogeneic PL treatment. The remaining PL were prepared and used to treat cartilage from the same horse, i.e. autologous. The PL were used to treat cartilage under pro-inflammatory environment as stated in Aim 1a. The effects of allogeneic vs autologous were assessed in cartilage and synovium as outlined in Aim1a.

Aim 3a. Is PL effect concentration dependent? PL treatments were prepared and utilized at different concentrations (PL:1.5x, 3x and 6x) and applied in cartilage under a pro-inflammatory environment as stated in Aim 1a. At the end of 10 days of experiment explants and medium were collected and analysis was performed as indicated in Aim 1a.

- CS:

Aim 1b. Does freeze-drying CS alters its protective effects in synovium or cartilage compared to its frozen preparations? CS treatments (fresh, freeze-dried, filtered-freeze-dried) were prepared and utilized at different concentrations (10% and 30%) in the presence of either cartilage or synovium explants that were stimulated with IL-1ß. At the end of 10 days of experiment explants (cartilage and synovium) and medium were collected for analysis. In cartilage, sixteen hours prior to treatment, $^{35}SO_4$ was added to the medium to be incorporated into the explants. At the end of the experiment, total GAG and $^{35}SO_4$ labeled GAG left in matrix and released in media were quantified using DMMB assay and Alcian blue respectively, as indicators of cartilage matrix degradation. In addition, mRNA expression of pro-inflammatory cytokines was used to evaluate the modulatory effects in inflammation for both cartilage and synovium.

Aim 2b. Can allogeneic CS be used in place of autologous? CS produced were pooled together to create the allogeneic CS treatment. The remaining CS were prepared and used to treat cartilage or synovium from the same horse, i.e. autologous. The CS were used to treat cartilage or

synovium (CS experiment) under pro-inflammatory environment as stated in Aim 1b. The effects of allogeneic vs autologous were assessed in cartilage and synovium as outlined in Aim1b.

Aim 3b. Is CS effect concentration dependent? CS treatments were prepared and utilized at different concentrations (10% and 30%) and applied in either cartilage or synovium explants under a pro-inflammatory environment as stated in Aim 1b. At the end of 10 days of experiment explants and medium were collected and analysis was performed as indicated in Aim 1b.

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CHAPTER 2

Evaluation of allogeneic freeze-dried platelet lysate for the prevention of pathologic changes in cartilage exposed to inflammatory conditions

1. Introduction

Osteoarthritis (OA) is one of the most important causes of equine and human musculoskeletal disability. The complete pathogenesis is still poorly understood, but loss of homeostasis in favor of catabolic activities contributes to the progressive degeneration characteristic of OA [1].

Current medical treatments for OA target mainly the symptoms, rather than modifying the disease progression. Therefore, there is a demand for potential disease modifying treatments. OA disease involves the interaction of numerous mediators that are necessary for cartilage homeostasis. Thus, regenerative therapies such as platelet-rich plasma (PRP) and platelet-lysate (PL- lysed PRP) have both experimental and anecdotally been reported to offer a novel treatment option for OA based on the delivery of multiple bioactive factors [2] [3].

PRP has shown promising results treating early osteoarthritis in human patients [4], demonstrating analgesic and antiinflammatory properties [5]. The intra-articular use of PRP in a preliminary non-controlled study involving patients with hip OA demonstrated to promote pain relief and improve joint function [6]. Additionally, in a prospective randomized double-blinded controlled trial, PRP was as efficient as hyaluronic acid (HA) injections for the treatment of knees with symptomatic OA [7]. The clinical improvement in patients treated with PRP is believed to be explained by the action of growth factors release by activated platelets. Growth factors including transforming growth factor ß (TGF-ß), platelet-derived growth factor (PDGF)

and connective tissue growth factor (CTGF/CCN2) are known to modulate tissue inflammation and healing (reviewed via Maia et al, 2009[8]). Through anabolic effects and the inhibition of metalloproteinase, platelet products may mediate and promote cartilage healing [9]. Further these effects may provide both symptom and disease modifying *in vivo* effects for OA patients [7, 9].

Although the positive clinical results support the use of PRP, questions related to its application remain to be answered. Those include optimal preparation methods, dose, treatment timing and frequency of application [10, 11]. Because PRP is a blood derived product, it presents variability between samples which leads to inconsistent results in tissue healing. Moreover, the necessary storage at low temperatures to preserve PRP properties is a challenge to many clinics and field practitioners.

Based on these issues, this study proposed a creation of a freeze-dried allogeneic PL for the delivery of disease modifying factors into the site of the lesion. By freeze-drying PRP the product would enable the availability for immediate use, and therefore, provide more convenient and practical option to deliver growth factors to the joint. In addition, pooling platelet derived products from different patients would allow a formation of a more homogeneous product for use.

The objective of this research is to investigate an off-the–shelf PL in either autologous and allogeneic format and compared to the current state of practice (in this experiment represented by frozen PL). We based this study in three hypotheses; 1) Freeze-dried PL will have similar effects compared to frozen preparations in cartilage stimulated with interleukin-1β (IL-1β). 2) Allogeneic PL will have similar effects compared to autologous preparations in cartilage stimulated with IL-1β. 3) PL effect will be concentration dependent in cartilage stimulated with IL-1β.

2. Material and Methods

The in vivo equine model offers extensive experience with clinical OA and is a consistent predictable model that can enable the observation of early pathological events and define therapies[12]. In addition, the horse is a target species for the use of various therapies to treat OA. Based on the significance of the equine model used in in vivo studies, the horse was used for collection of samples in this in vitro study.

Blood was collected from eight skeletally mature and healthy horses (2 -5 years of age). The procedures were approved by the Animal Care and Use Committee, protocol number 12-3879. After blood collection, horses were euthanized for reasons non-related to this study and cartilage explants were harvested.

2.1 PL preparation

To produce the PL, blood was collected from the horses and placed in 450 mL blood bags containing 63 mL of anti-coagulant citrate phosphate dextrose adenine (CPDA). The blood was then placed into 50 mL conical tubes and centrifuged at 200 xg for 18 minutes, to remove red and white cells while leaving the platelets in the plasma to create our baseline platelet product, denoted in this study as 1x PRP [11]. A small aliquot of the 1 x PRP was used for manual and automatic platelet counting (Siemens Advia 120 Automated Hematology analyzer). The platelet products were further centrifuged for 10 minutes at 1000 xg to pellet the platelets. The supernatant (platelet-poor plasma) was collected and all platelet pellets were frozen at -80^{o}C for storage as well as activation of the platelets [13].

Since the effects of PRP can potentially be influenced by platelet concentration, the platelet average of all 1 x PRP (baseline PRP) collected as well as the standard deviation were calculated and used to estimate the baseline platelet concentration used in this study. Based on

the baseline concentration defined, the pellets frozen from each horse were thaw and re-suspended in different volumes of fresh medium (described bellow) to reach the concentrations desired for the experiment. Thus, platelet products from all different horses had theoretically similar platelet counts, enabling better comparison between the different subjects of the study.

Half of the pellets were lyophilized for at least 18 hours (freeze –dried PL) and then frozen until the commencement of experiment while the frozen PL remained stored at -80°C. The platelet pellets from each horse were re-suspended in media [Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 1 % volume/volume of ITS Premix (6.25 mg of insulin, 6.25 mg of transferrin and 6.25 μg of selenium acid, 1.25 g of Bovine Serum Albumin and 5.35 mg of linoleic acid -BD Bioscience), 0.1 mM nonessential amino acids, 10 mM HEPES, 0.4 mM proline, 0.11 mM ascorbic acid, penicillin (100 U/mL) and streptomycin (100 μg/mL)] in different volumes to create products 1.5 x, 3 x and 6 x more concentrated then the baseline platelet count. The effects of different dilutions of PL, was designated in this experiment as Concentration.

The filtered freeze-dried PL treatment was processed similarly as described for the freeze-dried PL, however the product was submitted to filtration using a 33mm syringe low binding filter with PES membrane (45 μm), previously to lyophilization to remove platelets debris. The expectation was to leave only growth factors within the media after filtration. This treatment was included to verify if besides the growth factors, the platelets (or platelet debris) could have a participation in PL effect. The effects of different PL formulations (frozen, freeze-dried and filtered freeze-dried) was designated in this experiment as Treatment.

Finally, all the PL treatments and concentrations were tested in two different conditions; autologous and allogeneic. For the autologous treatment, the platelet pellets were diluted in media as mentioned and applied directly into the cartilage cultures of the same horses the blood for the PL production was collected (Figure 2.1).

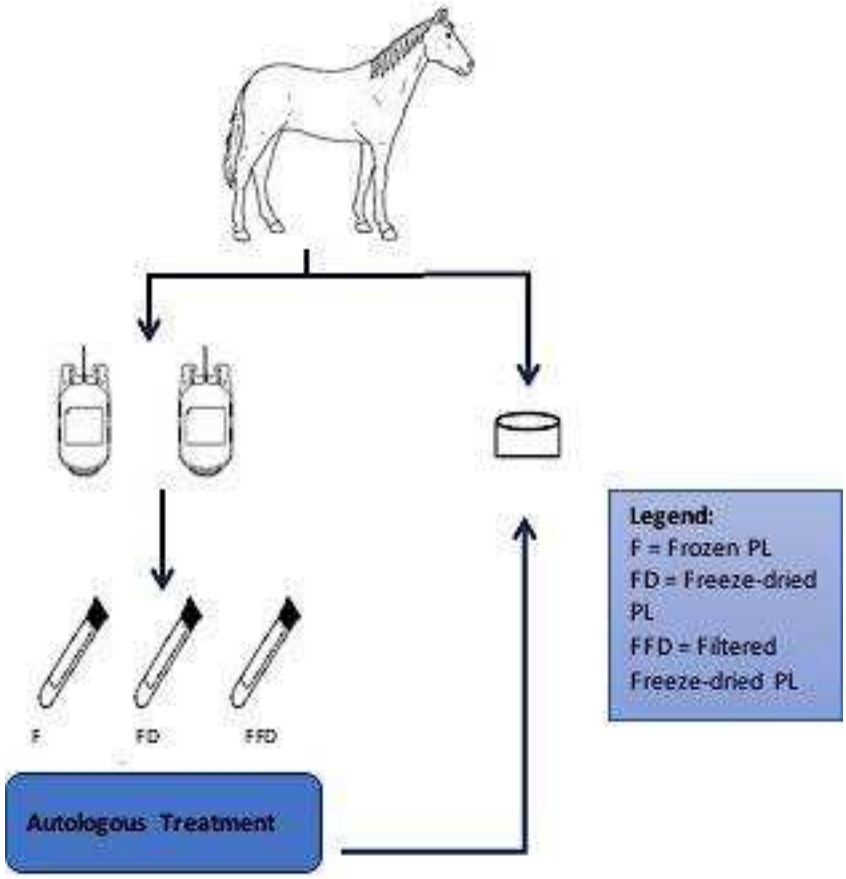

Figure 2.1: Autologous PL treatment used in the experiment. Blood was collected from 8 horses and used to process the PL. The PL was applied into the cartilage of the same horse the blood was collected.

On the other hand, for the allogeneic PL group from 4 horses were combined (2 groups of 4, N=8 horses) and applied in cartilage cultures from different horses (Figure 2.2). The effects of different conditions were designated in this experiment as Allogenicity.

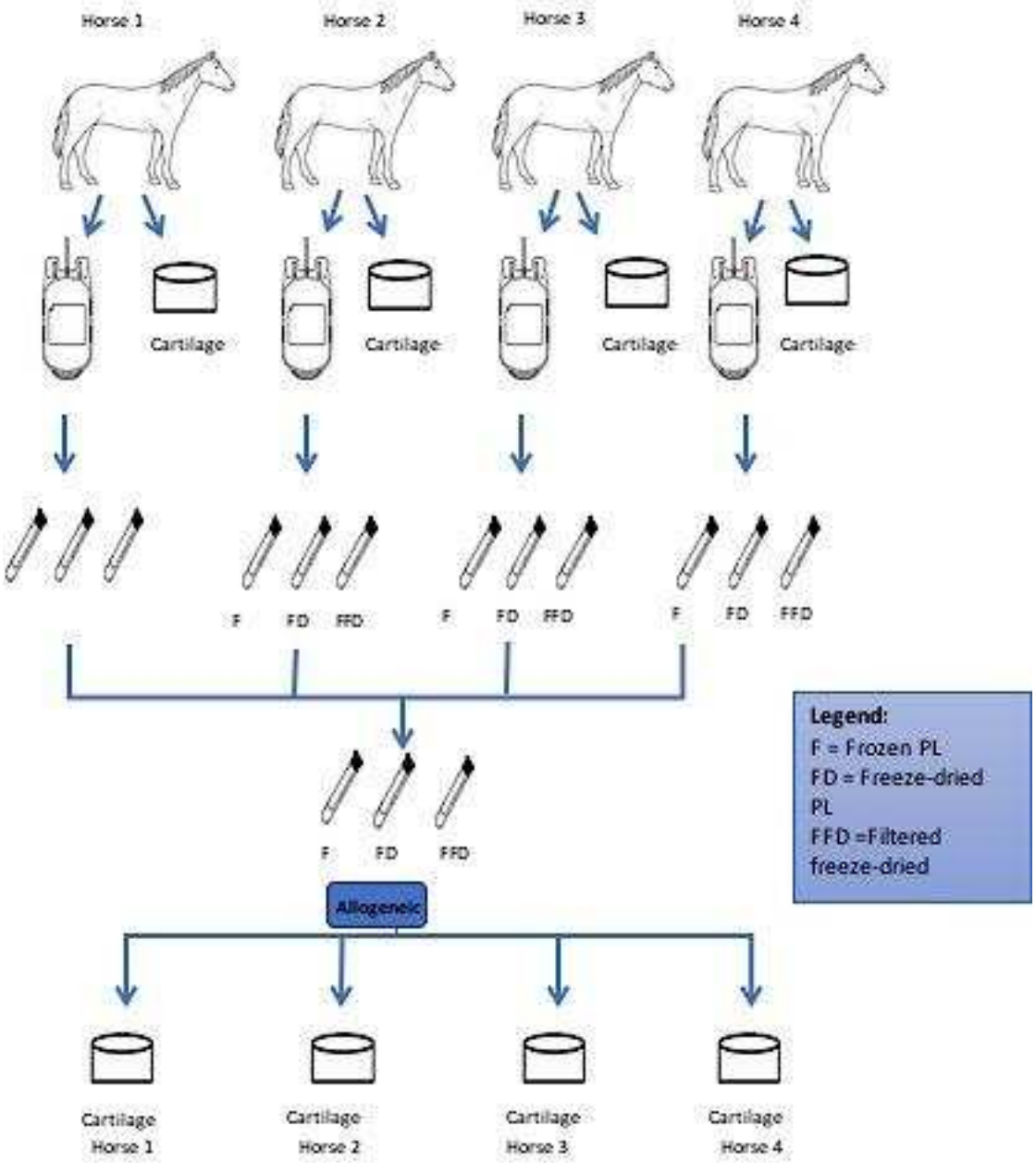

Figure 2.2: Allogeneic PL treatment. Blood was collected from different horses to produce the PL. The PL produced was combined and applied to cartilage from different horses.

2.2 Cartilage explant harvest

Immediately after euthanasia, cartilage from the stifle joint was harvested from each horse, using an 8mm punch to obtain explants with wet weights (ww) between 70-100 mg. The explants were placed in fresh sterile media and in ice immediately after harvest. Then, samples were placed in a 24 well plate and received fresh complete medium (same as described for platelet pellet dilution).

Although fetal bovine serum (FBS) is widely adopted in tissue culture, it's growth factors and other components could potentially interfere with the effects of the PL applied in this experiment. For this reason, the authors in this study opted for a serum-free medium, using ITS as media supplementation[14-16]. Yet, FBS has been used as the main media supplement in similar models as the one implemented in this study. Thus, to validate the efficiency of the model in comparison with previous findings, an FBS control group was used as well as the ITS controls.

In this experiment, the samples that were assigned to be treated with PL as well as the ITS control samples, received medium supplemented with 1%ITS. The samples that were randomly assigned to be the FBS control samples, received medium supplemented with 10% FBS, instead of ITS. Samples were then allowed to equilibrate in the designated medium for 48 hours in humidified incubator at 37°C before the commencement of the study.

2.3 Cartilage culture

Before application on cartilage explants, PL pellets were thawed and diluted in media in the different concentrations described. Autologous PL was applied on cartilage from the same horse the blood was collected and allogeneic PL was pooled together and applied in cartilage explants from different horses. Samples for all PL interactions were done in duplicate. The control samples used in this experiment (supplemented with ITS and FBS) were divided in two

groups; explants exposed to IL-1β (ITS+IL-1 β and FBS+IL-1 β) and non-exposed to IL-1β (ITS and FBS). The main effect of the two types of media supplementation used in this study (ITS and FBS) were designated as MEDIA, and the main effect of the presence or absence of IL-1β was denominated as IL-1β. A diagram of the PL groups and controls used in this experiment are presented in Figure 2.3.

At the commencement of the experiment (Day 0), cartilage explants were exposed to both interleukin-1 β [IL-1ß, 10 ng/mL[11, 17] (R&D Systems) diluted in 0.1% bovine serum albumin (BSA, Sigma Chemicals) and PBS] and the appropriate PL (with heparin 1 U/mL to avoid coagulation). Explants were submitted to a second dose of IL-1ß and PL treatment on day 4 of the experiment. At all the other days of the study, the explants received media only (without treatment). On the 10th day of the experiment all the cartilage explants as well as the media were collected and frozen in a -80ºC until analysis. A timeline of the study is outlined on Figure 2.4.

2.4 Biochemical analysis

2.4.1 Cartilage explant digestion

Cartilage explants were weighed then lyophilized to extract the moisture. Wet and dry weights were recorded and the samples were papain digested overnight at 60°C using a crystallized papain suspension[18].

2.4.2 Explant DNA quantification

DNA content in cartilage explants was determined using a fluorescent dye based assay (Hoechst 33258)[19]. In brief, digested cartilage explants were added to a diluted dye/buffer solution, placed in duplicates, into a 96 well plate. The Hoechst dye interacts with the DNA in the digested samples resulting in a change in color. The change in color was analyzed in this experiment through spectrophotometry.

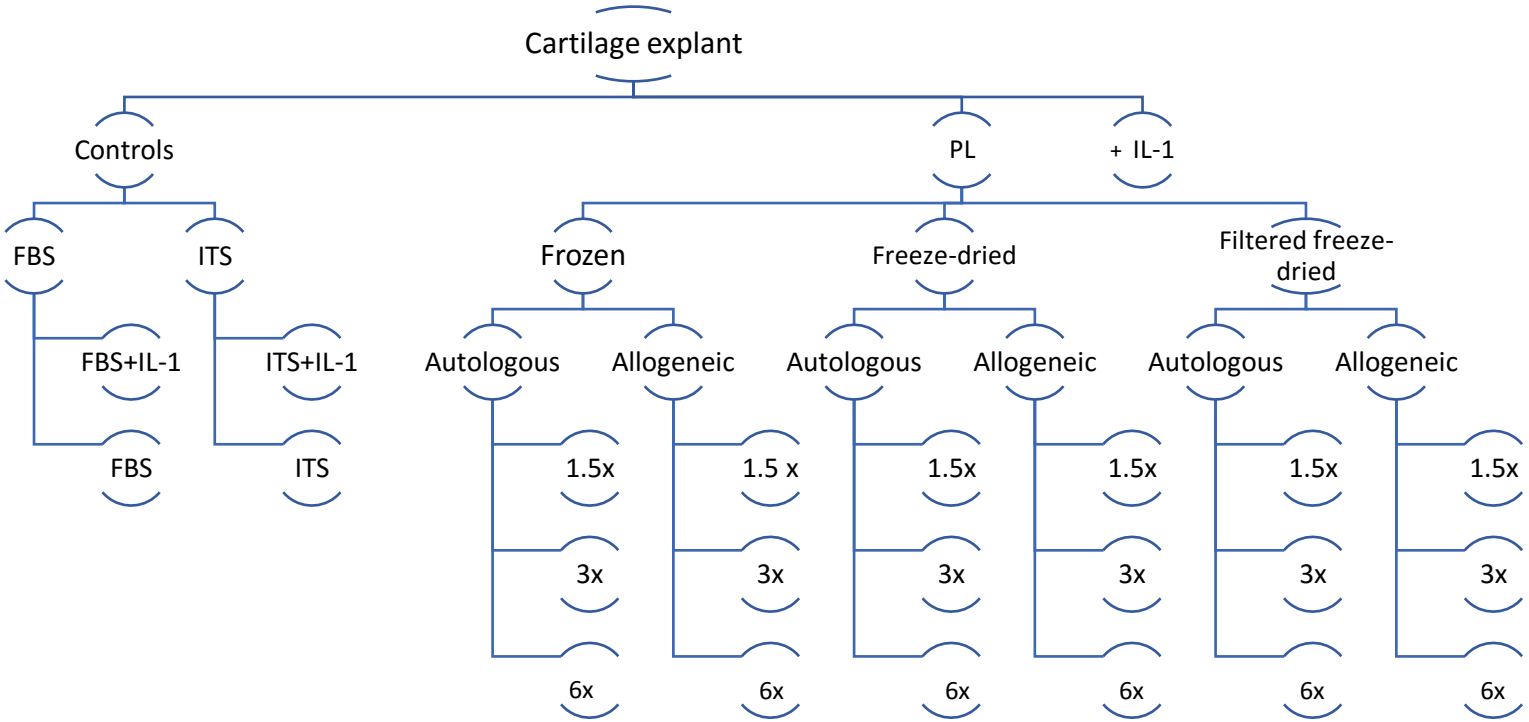

Figure 2.3: Diagram of PL groups and controls used in this study.

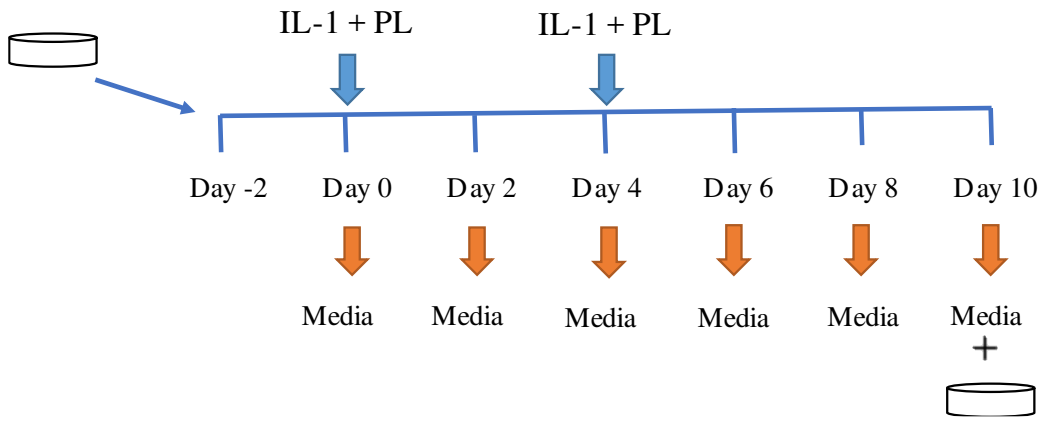

Figure 2.4: Timeline of the study. Cartilage samples were collected and let to equilibrate in media for 48 hours before the commencement of the experiment. Then, at day 0 and 4 samples were exposed to IL-1β and PL. All the other days of the experiment explants received full growth media only. Media was collected every-2 days and at the end of the study, cartilage and media samples were collected.

The samples were read against a standard curve using calf thymus DNA on a plate reader (Molecular Devices Spectramax M3). Samples were measured using a fluorometer with excitation of 360 nm and emission reading at 460 nm. The change in color intensity identified is correlated to the concentration of DNA found in the sample. Samples and standards were run in duplicate. DNA content within the samples was evaluated and used for normalization of the biochemical data in this study (total GAG in media and cartilage, and $^{35}SO_4$-labeled GAG in cartilage).

2.4.3 Explant glycosaminoglycan (GAG) quantification

Cartilage explants and media were analyzed for total GAG content using a modified method of the dimethyl-metilene blue (DMMB) assay[20]. In brief, the digested samples were added to a dye/buffer solution and read against a standard curve using chondroitin sulfate C from shark cartilage (Sigma). The DMMB dye binds to sulfated glycosaminoglycan, forming complexes that result in change of color within the sample. The color was measured using

spectrophotometry, and samples were read at 530nm in the plate reader Molecular Devices Spectramax M3. The change in color intensity is correlated to the amount of glycosaminoglycan found in the sample. All samples and standards were run in duplicate in this assay. GAG was normalized to DNA content in cartilage and presented as GAG in μg / μg of DNA.

2.4.4 Explant $^{35}SO_4$-labeled proteoglycans quantification

In this study $^{35}SO_4$-labeled GAG in cartilage was measured at the termination of the study as an indirect indicator of newly synthetized GAG degradation. After stimulation with IL-1β during the experiment inducing cartilage matrix degradation, $^{35}SO_4$-labeled GAG left in cartilage was measured. Thus, an increased level of $^{35}SO_4$-labeled GAG present in cartilage would be interpret as less matrix degradation.

Sixteen hours prior to Day 0, 5 μCi of $^{35}SO_4$ was added to the media of all cartilage explants. The media and isotope were removed and explants washed before stimulation with IL-1β and PL treatment. Upon completion of the experiment the concentration of $^{35}SO_4$ present in the explant was measured using a modified Alcian blue system. In brief, samples were added in duplicate, to each well of a Multiscreen 96 well plate, along with assay buffer and Alcian blue dye. The multiscreen plates were attached to a vacuum manifold which draw the solutions through the screen allowing only the radiolabel bound to GAG and Alcian blue to be catch in the screen. The screen was then analyzed by liquid scintillation counting. For the standards, solutions containing different concentrations of $^{35}SO_4$ were also analyzed via liquid scintillation counting[21]. Samples and controls were run in duplicate. Data was normalized to DNA content and presented as DPM/μg of DNA.

2.5 Gene Expression

Quantitative PCR was performed in cartilage explants at the end of the experiment to evaluate gene expression of catabolic cytokines and enzymes related to OA to access the effects of PL as an anti-inflammatory treatment.

2.5.1 Cartilage RNA extraction

Cartilage explants were collected from culture, weighted and immediately placed in Tryzol reagent (Invitrogen) and stored at -80°C until analysis. For RNA extraction, samples were thaw and placed in a microcentrifuge tube for pulverization for 30 seconds using a BioSpec 31100B X Mini-BeadBeater-1. Immediately after pulverization, Tryzol was added and samples were homogenized, incubated at room temperature and centrifuged for 10 minutes at 12,000 xg. Supernatant was collected and nucleic acids were extracted with the addition of 20% chloroform (volume/volume) and centrifuged for 15 minutes, at 12,000 xg. This step was repeated as necessary to ensure separation of nucleic acids from extra-cellular matrix. Nuclei acids were then precipitated with isopropanol and centrifuged for 10 minutes at 10,000 xg. All centrifugations were performed at 4°C. After the last centrifugation, the nucleic acid pellets were then resuspended in nuclease-free water and absorbance was measured at 260/280 nm to determine nucleic acid yield and purity.

2.5.2 Reverse-transcription

After extraction, RNA was treated for genomic DNA contamination and reverse-transcribed simultaneously using a blend of oligodT and random primers (iSript^{TM}gDNA Clear cDNA synthesis kit, Bio-Rad). DNase treatment was done for 5 minutes at 25°C and DNase inactivation was performed for 5 minutes at 95°C. Reverse-transcription protocol was performed

as follow: priming for 5 minutes at 25°C, reverse-transcription for 20 minutes at 46°C and enzyme inactivation for 1 minute at 95°C.

2.5.3 Quantitative Polymerase Chain Reaction (qPCR)

cDNA solution and iTap^{TM}Universal Syber ®Green Supermix (Bio-Rad, Hercules-CA) were mixed with primers for the genes of interest and gene expression levels were determined by quantitative real-time PCR utilizing a CFX96 Touch^{TM}Real-Time PCR Detection System (Bio-Rad, Hercules-CA). Conditions were: initial activation step, 3 minutes at 95°C, denaturation 10 seconds at 95°C, annealing 30 seconds at 60°C and melting temperature 65°C-95°C, incrementing 0.5°C for 0.5 second. Expression levels of IL-1ß, MMP-1, COX-2, ADAMTS-4, ADAMTS-5 and Ubiquitin C (UBC) were evaluated in the cartilage and synovium samples. All primers were designed using the National Center of Biological Information (NCBI) and PrimerQuest Tool (from Integrated DNA Technologies-IDT) software. Characteristics used for primer designed were; size under 150 nucleotides, should span in an exon-exon junction, CG% close to 50%, melting temperature close to 60°C and the minimum of hairpins or secondary structures. Then, efficiency curves were performed using 10-fold dilutions of cDNA from equine chondrocytes stimulated with IL-1β. If the efficiencies were bellow 90%, PCR products were run in a 2% agarose gel to confirm the right sequence was amplified. Primer sequence used are presented in the table below (Table 2.1).

Table 2.1: Sequences of the primers used in the study.

Primers used in the study	Primers sequence (5' to 3')
ADAMTS-5	Forward: AAGGTGACTGATGGGACCGAATGT
	Reverse: TTTGAGCCAATGATGCCGTCA
ADAMTS-4	Forward: GTCCCAAAGGCCACATACTT

	Reverse: GCCACTCTTACTTGCCCATATC
MMP-1	Forward: ACTGCCAAATGGACTTCAAGCTGC
	Reverse: TCTTCACAGTGCTAGGAAAGCCG
IL-1β	Forward: CCAGAGGCGGCCGGGACATAAC
	Reverse: GGGAAGGCAGCTGGGCATTGATT
UBC	Forward: GGCTGTTAGCTTTTCAGTCTTGTC
	Reverse: CTTAAATTGGGGCTAATGGCTGG

Gene expression levels of the target genes were normalized to the ubiquitin C (UBC) as endogenous control (Δ_{CT}). Then, the Δ_{CT} generated was subtracted from the Δ_{CT} of the ITS+IL-1β controls, resulting in the $\Delta\Delta_{CT}$. Therefore, the gene expression in this study was presented in fold ($2^{-\Delta\Delta CT}$) relative to the ITS+IL-1β control. Since a Ct of 38.5 was still within a range for the efficiency curves developed for the primers of this study, samples with a Ct above 38.5 were not considered for analysis.

2.6 Data Analysis

In this experiment, three analysis were performed. The first analysis (analysis 1) was performed to evaluate the effect of IL-1β stimulation in cartilage. The second analysis (analysis 2) was performed to evaluate the effects of PL treatments compared to ITS control only, both in a stimulated environment with IL-1β. Finally, the third analysis (analysis 3) was performed to evaluate the effects of Treatment, Allogenicity and Concentration in PL treated samples in a stimulated environment with IL-1β.

All three analysis in this experiment were performed using a mixed-model analysis of variance. The GLIMMIX procedure in SAS 9.3 (SAS Institute, Carey, NC) was used. The media

GAG, explant GAG and $^{35}SO_4$-labeled GAG were considered as dependent variables. In addition, gene expression was considered dependent variable for analysis 3. Independent variables were described according to the analysis performed. Horse was defined as a random effect.

Analysis 1: the independent variables evaluated here were IL-1β and MEDIA. PL treated samples were not included in this analysis and only control samples were considered. The main effects of IL-1β and MEDIA as well as interactions were evaluated.

Analysis 2: the independent variable evaluated was Treatment. In this analysis, PL treated samples stimulated with IL-1β were compared to ITS+IL-1β control. Only the main effect of Treatment was considered.

Analysis 3: independent variables evaluated were Treatment (frozen, freeze-dried and filtered freeze-dried), Allogenicity (autologous and allogeneic) and Concentration (1.5x, 3x and 6x). Only PL treated samples were considered in this analysis. The main effects evaluated were Treatment, Allogenicity and Concentration as well as interactions.

Dependent variables were checked for normality based on student residual plots and when needed, normality was corrected by log transformation. Outliers were identified based in diagnostic residual plots. Values greater than +/- 3 standard deviation from the mean were excluded from the data set and statistical analysis was performed again. Restricted Maximum Likelihood (REML) was used as estimation technique in this experiment. Protection against multiple comparisons was achieved by using a protected F test. Individual comparisons supported by the F-test were done using least-squares means procedure. In all statistical comparisons, P value < 0.05 was considered significant.

For the results of the main effect or interactions, the highest order was reported, further only significant outcomes were reported. Values were represented in means ± standard error of the mean (mean ±SEM). In the graphs, bars represent standard error of the mean and different letters indicate statistical difference.

3. Results

3.1 PL Content

The average baseline automated platelet count was 273.25 x 10^3 platelets/μL (202-368 platelets/μL) for the 8 horses. Manual counting resulted in an average of 291.8 x 10^3 platelets/μL. For subsequent calculations, an average platelet count (based on the manual and automatic counts) was calculated and used for the 1x PRP estimation (282.52 platelets x 10^3 platelets/μL. Table 2.2). These counts are within normal range for the horse (125-300 x 10^3 platelets/μL) [1]. The mean platelet volume estimated was 7.975 femtolitre (6.9-9.4 femtolitre), which is within normal range for horses (5-10.5 femtolitre)[1]. Nucleated cells were counted using an automatic method. The average nucleated cell count was 0.8125 x 10^3 cells/ μL, which was below the normal range of horses (5.5-10.5 x 10^3 cells/μL)[1].

The average value estimated for platelet concentration after rehydration of the pellets for 1.5, 3 and 6 x PL dilutions were 423.78, 847.57 and 1,695.15 x 10^3 platelets/μL respectively. In a similar fashion the concentration for nucleated cell counts for the concentrated PL were 1.218 x 10^3 cells/μL, 2.4375 x 10^3 cells/μL and 4.875 x 10^3 cells/μL for 1.5 x, 3 x and 6x respectively.

[1]Values defined by normalization studies performed at the Veterinary Diagnostic Laboratory, Colorado State University.

Table 2.2: Average values obtained for each horse using an automated counting procedure. Values defined by normalization studies performed at the Veterinary Diagnostic Laboratory, Colorado State University.

Horse	Platelet count (10^3/µL)	MPV (10^3/µL)	WBC (10^3/µL)
LG1	259	7.3	0.4
LG6	368	9.1	0.4
LG 8	271	9.4	1
LG 12	248	7.7	0.9
LG 13	238	6.9	0.8
LG 15	268	6.9	1.4
LG 18	332	9	0.8
LG 19	202	7.5	0.8

All PL described here were considered above baseline in platelet concentration and bellow the baseline for WBC according to the PAW classification system [22] (See Appendix 1).

3.2 Evaluation of IL-1 β Stimulation in cartilage (analysis 1)

To verify the IL-1β effect in cartilage explants, control samples stimulated and non-stimulated with IL-1 β were compared. The outcome parameters considered were $^{35}SO_4$-labeled GAG and total GAG in cartilage explants as well as GAG in the media. The main effects evaluated were IL-1β and MEDIA, as well as the interaction MEDIA*IL-1β.

3.2.1 Media GAG

An increase in media GAG when IL-1β was present independent of media used (ITS or FBS) was observed. This observation was statistically significant at Days 2 (F test. P=0.0107) and 6 (F test, P=0.0226) when the main effect of IL-1β was assessed (Table 2.3). At days 4, 8 and 10 of the experiment a trend in increase of GAG was observed in media samples of stimulated explants, however no significance was observed (Table 2.3).

Table 2.3: GAG in media during the experiment for the control samples. Increased levels of GAG were observed in media samples of cartilage explants exposed to IL-1β. However, significance was only observed at days 2 and 6 of the experiment. Different letters denote statistical difference. Levels of significance 0.05.

Controls	Day 2 **(mean ± SEM)**	Day 4 **(mean ± SEM)**	Day 6 **(mean ± SEM)**	Day 8 **(mean ± SEM)**	Day 10 **(mean ± SEM)**
ITS	38.19 ±17.76^{ab}	49.60 ± 27.72	26.13 ±20.12^{ab}	40.23 ± 29.23	28.89 ± 28.89
ITS+IL-1β	74.28 ± 17.03^{a}	60.52 ± 26.3	54.95 ± 17.92^{ab}	43.69 ± 26.05	27.2 ± 27.2
FBS	33.00 ± 17.81^{b}	44.83 ± 26.3	9.11 ± 20.21^{b}	93.52 ± 27.48	86.84 ± 28.93
FBS+IL-1β	68.58 ±17.82^{ab}	65.73 ± 27.84	62.32 ± 20.21^{a}	57.70 ± 27.48	44.80 ± 28.93

3.2.2 Total GAG in cartilage

At the end of the study, total GAG in cartilage for the main effects of IL-1β and MEDIA did not demonstrate to be significant.

3.2.3 $^{35}SO_4$-labeled GAG in cartilage

Although samples stimulated with IL-1β had less $^{35}SO_4$-labeled GAG content in cartilage (ITS+IL-1β: 53.56 DPM ± 19.68, FBS+IL-1β: 37.10 DPM± 22.73) compared to non-stimulated sample explants (ITS: 74.92 DPM ± 22.73, FBS: 78.9957 DPM ± 21.04) the difference was not significant.

3.3 PL treated samples compared to controls (analysis 2)

To study the effects of PL in cartilage under a pro-inflammatory environment, PL treated samples exposed to IL-1β were compared to ITS+IL-1β controls. The outcome parameters measured were total media GAG and explant total GAG and $^{35}SO_4$-labeled GAG.

The statistical model described in data analysis section was considered and only the main effect of Treatment was assessed.

3.3.1 Media GAG

No significant differences were observed for levels of GAG in media between PL treated samples and ITS+IL-1β control at any days of the experiment.

3.3.2 Total GAG in cartilage

Total GAG found in cartilage explants treated with PL were not significantly different compared to both ITS+ IL-1β control (Figure 2.5).

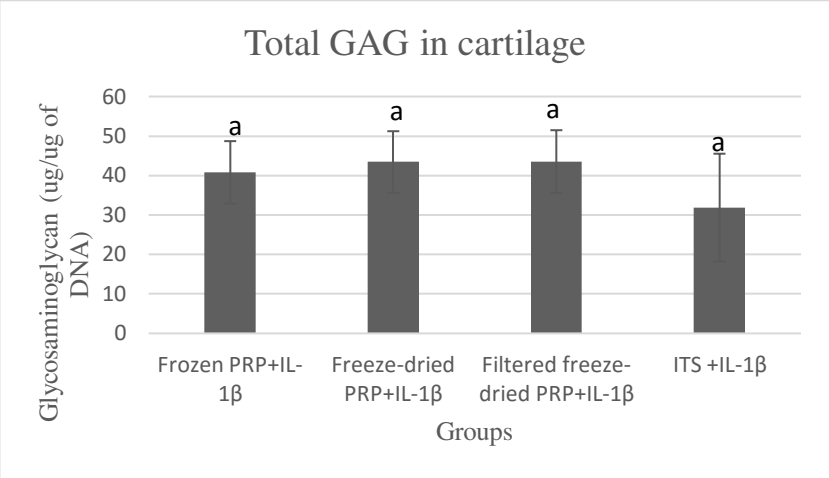

Figure 2.5. Total GAG in cartilage for PL treated groups and ITS+IL-1β control. No significant difference was observed for samples treated with PL and ITS+IL-1β control in cartilage at the termination of the study. Level of significance 0.05.

3.3.3 $^{35}SO_4$-labeled GAG in cartilage

$^{35}SO_4$-labeled GAG measured in cartilage explants at the end of the experiment was not different comparing samples treated with PL and ITS+ IL-1β control (Figure 2.6).

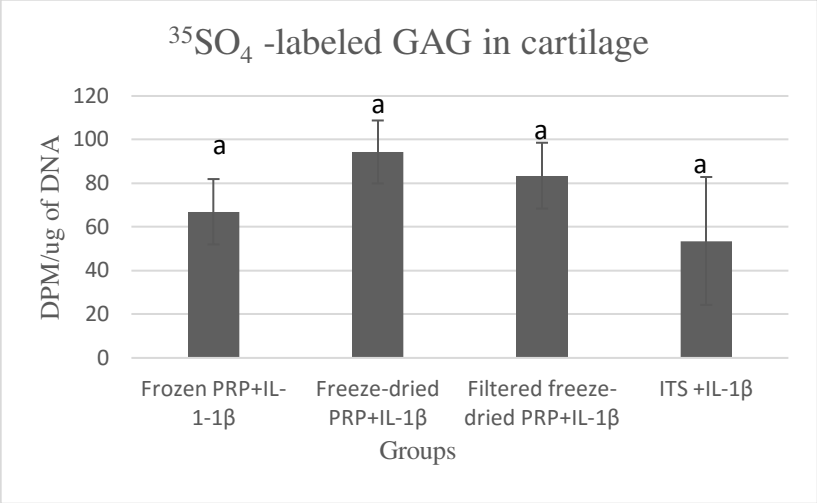

Figure 2.6. Total $^{35}SO_4$ -labeled GAG in cartilage for PL treated groups and ITS+IL-1β control. No significant difference was observed for samples treated with PL and ITS+IL-1β control in cartilage at the termination of the study. Level of significance 0.05.

3.4 Study of PL effects (analysis 3)

To address the hypotheses of this study, the main effects of PL Treatment, Concentration and Allogenicity as well as their interactions for total media GAG and cartilage GAG and $^{35}SO_4$ -labeled GAG were assessed. For these comparisons, only PL treated samples were considered.

3.4.1 Media GAG

At Day 10, a significant interaction was observed between PL Treatment*Allogenicity for total media GAG (F test. P=0.0549). Specifically, significantly more GAG was observed in the media for the autologous filtered freeze-dried PL when compared to allogeneic filtered freeze-dried and autologous frozen and freeze-dried PL treated samples (Figure 2.7).

While not supported by the protected F-test (2-way interaction was not statistically significant) the increase in media GAG at Day 10 for filtered freeze-dried PL treated samples appeared to be the result of the 1.5X concentration. Filtered freeze-dried PL treated samples had significantly more GAG in media at day 10 compared to both frozen and freeze-dried autologous and filtered freeze-dried allogeneic treated samples (Figure 2.8).

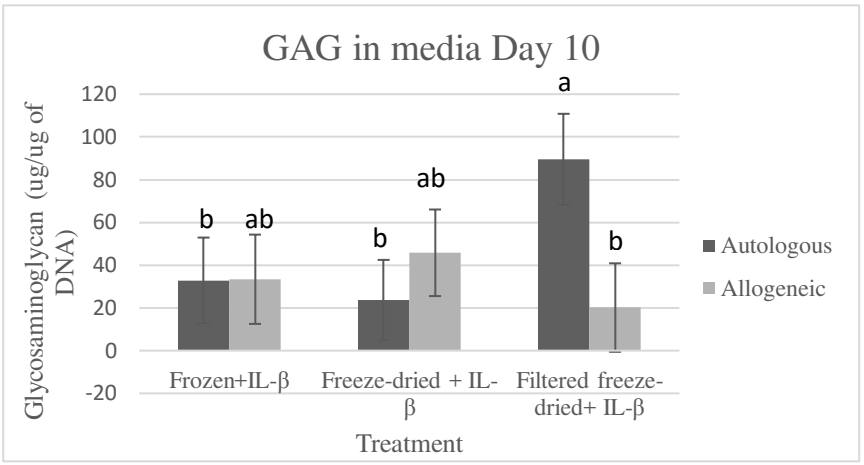

Figure 2.7: Mean media GAG at Day 10 plotted by PL Treatment*Allogenicity interaction. Autologous filtered freeze-dried PL had significant increase GAG in media at Day 10 compared to its allogeneic condition, autologous frozen and freeze-dried PL. Level of significance 0.05.

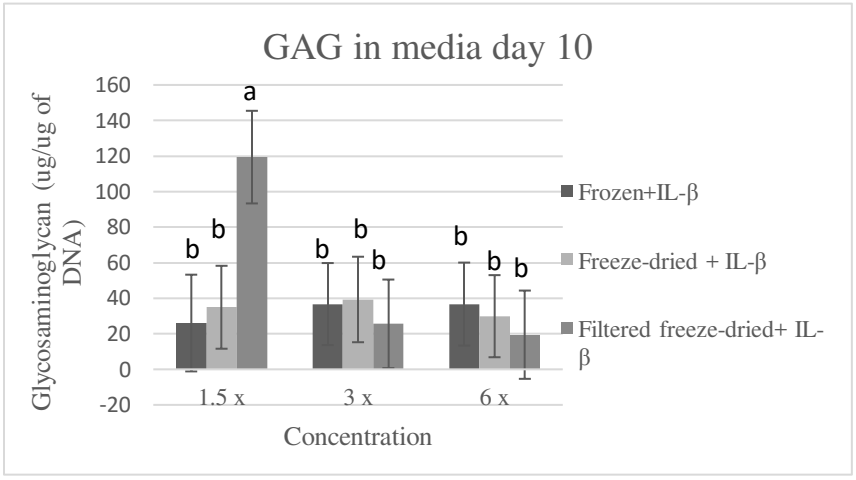

Figure 2.8: Media GAG plotted by PL Treatment*Concentration interaction. Frozen 1.5 x PL treated samples released significantly greater concentrations of GAG compared to all the other Treatment and Concentration interactions. Levels of significance 0.05.

3.4.2 Total GAG in Cartilage

When comparing the GAG in cartilage explants taking into consideration PL main effects and interactions, no significant difference was observed.

3.4.3 $^{35}SO_4$-labeled GAG

Concentration main effect was significant for $^{35}SO_4$-labeled GAG in cartilage at the termination of the study (F test. P=0.0127). Samples treated with 3x PL demonstrated significantly more $^{35}SO_4$- labeled GAG retained in cartilage compared to samples treated with 1.5x and 6x PL (P=0.0065 and P=0.017, respectively). Considering individual comparisons, autologous 3x PL demonstrated increased levels of $^{35}SO_4$-labeled GAG in cartilage compared to autologous and allogeneic PL in both 1.5 x and 6 x concentrations (Figure 2.9). As such, freeze-dried 3x had increased levels of GAG in media compared to freeze-dried, frozen and filtered freeze-dried PL at 1.5 and 6x concentrations (Figure 2.10). The significant effect observed in these comparisons for $^{35}SO_4$-labeled GAG appeared to be a result of the 3x concentration (Figure 2.11).

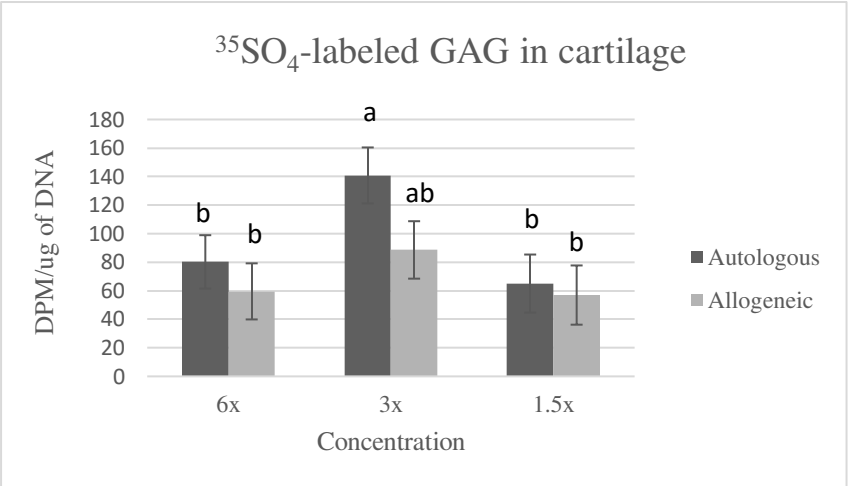

Figure 2.9: $^{35}SO_4$-labeled GAG in cartilage plotted by Allogenicity*Concentration interaction. Samples treated with autologous 3x PL demonstrated increased levels of $^{35}SO_4$-labeled GAG in cartilage compared to autologous and allogeneic combinations of 1.5x and 6x concentrations. Level of significance 0.05

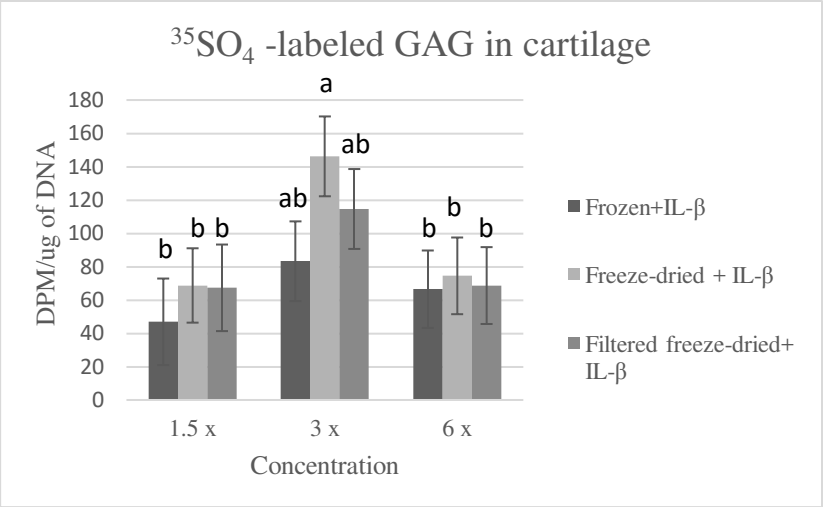

Figure 2.10: $^{35}SO_4$-labeled GAG in cartilage plotted by Treatment*Concentration interaction. Freeze-dried 3x treated samples demonstrated significantly increase of $^{35}SO_4$-labeled GAG in cartilage compared to frozen, freeze-dried and filtered freeze-dried in 1.5x and 6x concentrations. Level of significance 0.05.

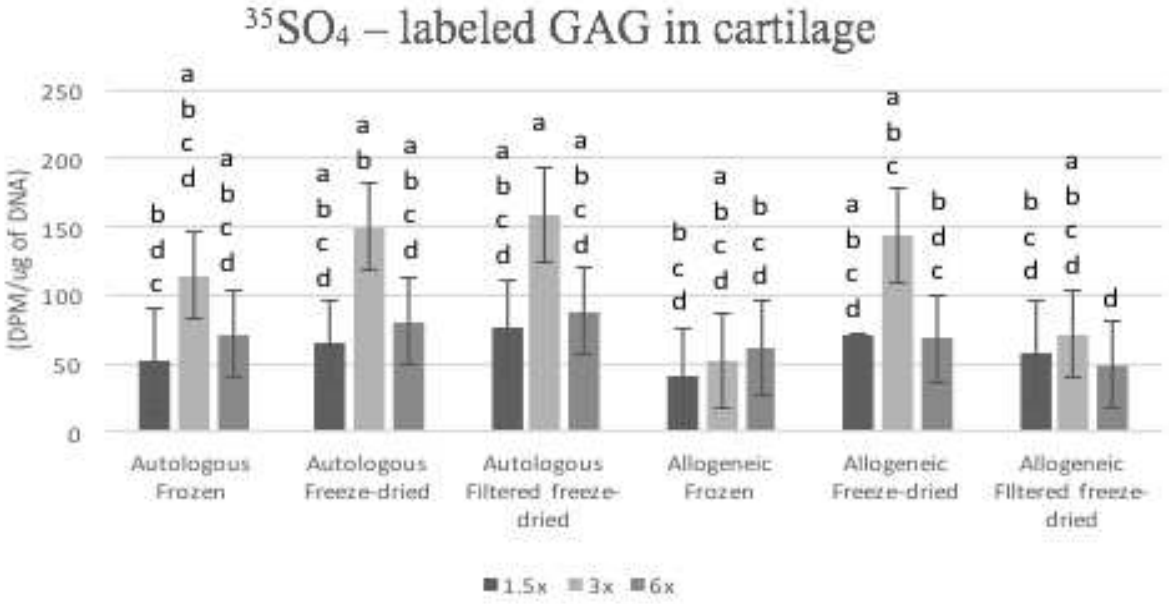

Figure 2.11: $^{35}SO_4$ -labeled GAG in cartilage plotted by Treatment*Allogenicity*Concentration interactions. With exception of allogeneic frozen, in all the other interactions the 3x concentration had more $^{35}SO_4$-labeled GAG in cartilage at the termination of the study. Level of significance 0.05.

3.4.4 Gene Expression

Gene expression of catabolic cytokine and enzymes related to OA were accessed here to verify the potential modulatory effect of PL. To meet the assumption of normality the data was log transformed for statistical analysis, however for ease of presentation the raw fold changes are presented.

3.4.4.1 ADAMTS-5

Significant upregulation in fold change relative to ITS+IL-1β was observed in samples treated with frozen PL in comparison with samples treated with freeze-dried PL (about 11.35-fold difference, F test P=0.0009). Additionally, significant effect of Treatment*Allogenicity interaction was observed for ADAMTS-5 gene expression (F test, P=0.0158). Autologous frozen PL (21.55 ±21.73) had significantly more gene expression compared to autologous freeze-dried (0.98 ± 6.31) PL, allogeneic frozen (4.13 ± 1.21) and freeze-dried PL (1.62 ± 0.87. P=0.0057-0.0002).

3.4.4.2 ADAMTS-4

Although strongly up-regulated relative to ITS +IL -1β control (up-to 114-fold), no significant difference was observed for the gene expression of ADAMTS-4 for the main effects of Treatment, Concentration or Allogenicity.

3.4.4.3 MMP-1

All PL treated samples showed upregulation of MMP-1 gene expression relative to ITS+IL-1β control. The main effect of Treatment was significant for expression of this gene (F test, P=0.0005).

There was a significant increase for samples treated with freeze-dried PL (7.1 ±1.99) compared to samples treated with frozen PL (4.46± 1.99) was observed (P=0.0005). No significant change in expression for this gene was seen for Allogenicity or Concentration main effects.

Individual interactions between Treatment*Allogenicity and Treatment*Concentration were significant for the expression of MMP-1 (Figure 12 and 13). Allogeneic freeze-dried PL induced significantly more expression of MMP-1 compared to allogeneic and autologous frozen (Figure 2.12). Moreover, freeze-dried 3x PL increased gene expression of MMP-1 significantly compared to freeze-dried 1.5x, and frozen at all concentrations (Figure 2.13). Such significant findings appear to be a result of freeze-dried PL.

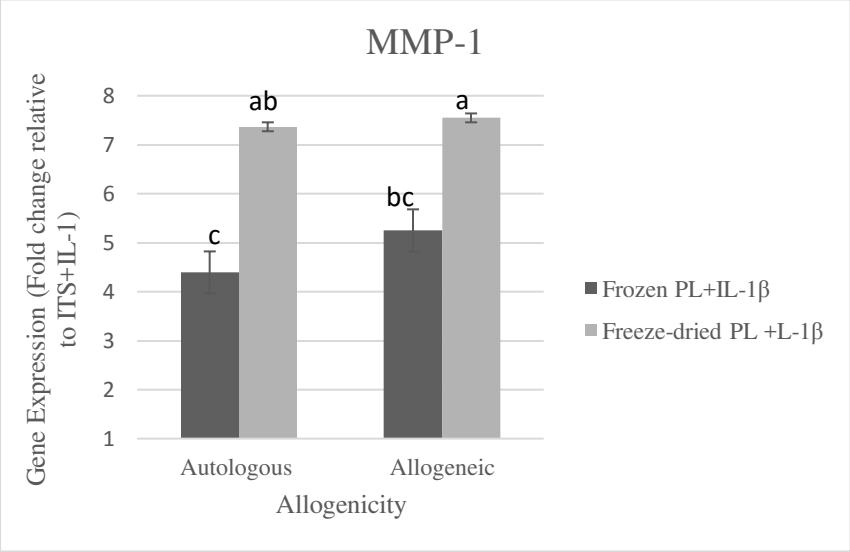

Figure 2.12: Gene expression of MMP-1 plotted by Treatment*Allogenicity interaction. Allogeneic Freeze-dried induced significantly more MMP-1 expression compared to Allogeneic Frozen and Autologous Frozen combinations. Level of significance used 0.05.

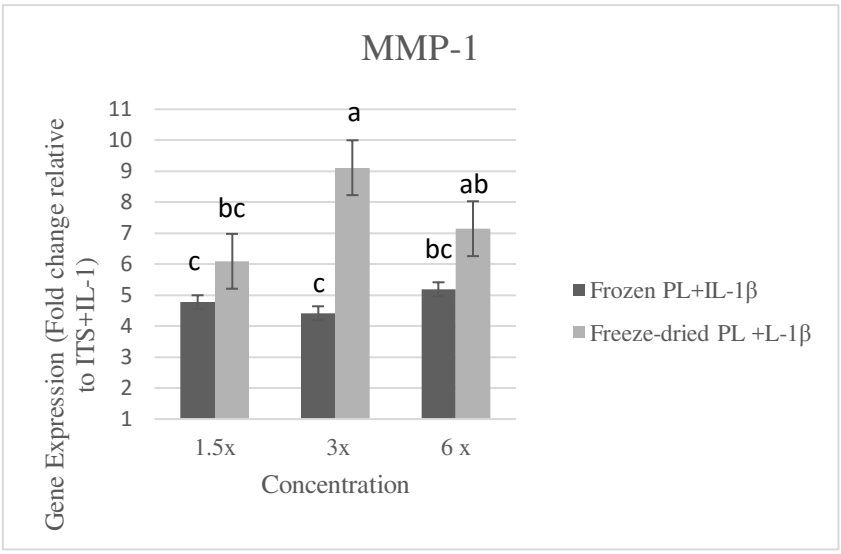

Figure 2.13. Gene expression of MMP-1 plotted by Treatment*Concentration interaction. Freeze-dried 3x PL induced significantly more MMP-1 expression compared to Freeze-dried 1.5x and Frozen in all concentrations. Level of significance used 0.05.

3.4.4.4 IL-1β

All PL treated samples presented upregulation of IL-1β in cartilage relative to ITS+IL-1β control. Allogenicity and the interaction Treatment*Allogenicity did demonstrate to have a significant effect in IL-1β gene expression (F test, P=0.0157 and P=0.0179, respectively).

Specifically, Autologous Frozen PL increased expression of IL-1β significantly compared to Allogeneic Frozen, Autologous Freeze-dried and Allogeneic conditions (Figure 2.14). No difference in gene expression of IL-1β was found for the different Concentrations of PL applied.

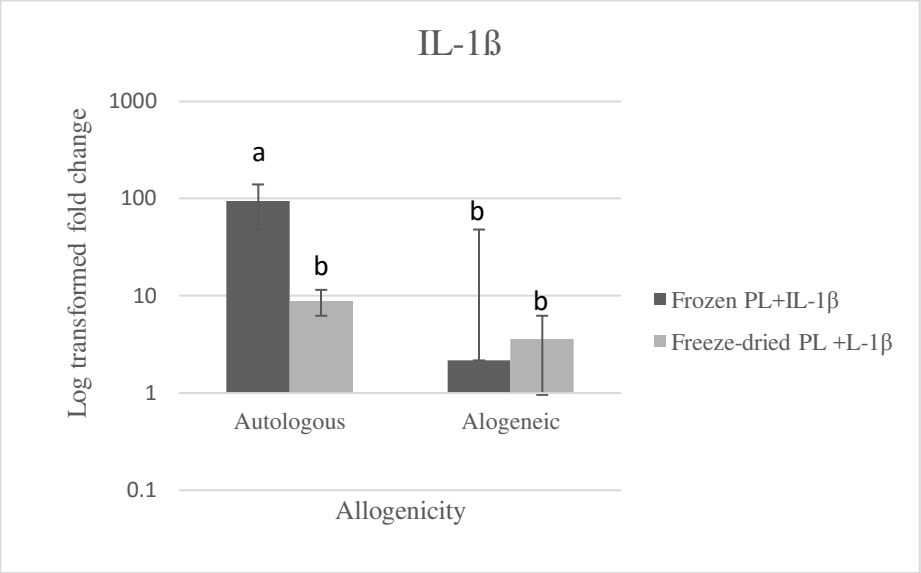

Figure 2.14: Gene Expression of IL-1β plotted by Treatment*Allogenicity interaction. Results presented in Log scale of the fold change relative to the ITS+IL-1β control for better visualization of the results. Autologous Frozen PL showed significantly increased gene expression of IL-1β compared to Autologous Freeze-dried and Allogeneic Frozen and Freeze-dried PL. Level of significance used 0.05.

3.4.4.5 COX-2

The gene expression of COX-2 relative to ITS-IL-1β was upregulated in all samples treated with different PL. Treatment main effect was significant in upregulating COX-2 expression in cartilage (F test, P=<0.0001) however, the difference between frozen and freeze-dried PL treated samples was less then 1-fold difference and therefore not biologically significant. The interaction of Treatment*Allogenicity as main effects was also significant (F test, P=0.0195). Allogeneic freeze-dried PL had a significant increase in gene expression of COX-2 compared to frozen in both autologous and allogeneic condition (Figure 2.15). The Allogenicity itself was not significantly different. Concentration and interactions did not present significance for the F test.

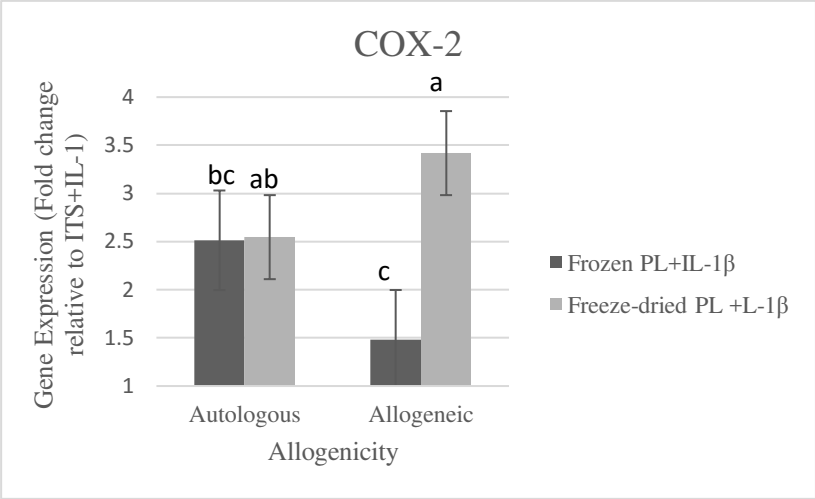

Figure 2.15: Gene expression of COX-2 plotted by Treatment*Allogenicity interaction. Cartilage samples treated with allogeneic freeze-dried PL demonstrated a significant increase in expression of COX-2 compared to samples treated with allogeneic frozen, autologous frozen and freeze-dried PL. Level of significance used 0.05.

4. Discussion

The concentration of platelets in the 1 x PRP produced in this study was within normal range for horses, according to normalization studies performed by the Veterinary Diagnostic Laboratories, at Colorado State University. The white blood cell content on the other hand, was below the baseline[1]. Once the platelet pellets were freeze-thawed and then rehydrated to achieve the platelet concentrations desired for PL in the study, the final concentration of platelets could not be confirmed and therefore, was estimated based on the counts of the 1xPRP. Still, the estimate platelet counts of the PL produced was equivalent to what was observed in previous studies using similar protocols [11]. In fact, platelet counts for 3x and the 6x PL have exceeded the concentrations of platelets from previous studies that used double spin protocols[11].

Indeed, according to the PAW classification system, all the PL described here would be considered above the baseline platelet count [22]. In relation to the WBC counts, all the PL

produced including the 6x concentration, were below the baseline according to the PAW classification system[22], and lower compared to other double spin methods previously described [11]. The presence of leukocytes in PRP is particularly controversial and many researchers consider the use of platelet-derived products rich in leukocytes potentially harmful because of the release of radical oxygen species (ROS), pro-inflammatory cytokines and degradative enzymes [23]. Neutrophils in particular have been associated with a potential decrease in healing [23]. In this study, the neutrophil concentration cannot be discussed because the differential count in white blood cells was not performed. Yet, the protocol applied in this experiment showed to produce an enriched platelet-derived product with leukocyte counts considered low compared to whole blood, which would be desirable to control for potential catabolic effects of platelet products [23].

Equine recombinant IL-1β was used in this experiment to induce a pro-inflammatory environment in cartilage explants in vitro [24]. When analyzing the main effect of IL-1β (Analysis 1), significant increase in media GAG was observed 48 hours after exposure to this cytokine at days 2 and 6 compared to non-stimulated control samples. The increase in media GAG is an expected result due to increase in catabolism caused by IL-1β. As one of the main cytokines in OA, IL-1β its known to increase expression and activity of metalloproteinases and agrecanases [24] [25] leading to progressive degradation of the matrix. For this reason, IL-1β has been successfully implemented in previous studies and in our laboratory as an in vitro model of OA[11].

The effect of IL-1β stimulation in cartilage explants with regard to total GAG and $^{35}SO_4$-labeled GAG was not significant at the termination of the study. These results are not in accordance with previous findings where cartilage was continuously exposed to IL-1β [17, 26]. In

this experiment an acute stimulation with this cytokine was used at Days 0 and 4. Such differences in experimental design could explain the lack of significance in cartilage at the termination of the study. However, considering the significant effect of IL-1β in media GAG at day 2 and 6 and the trend observed in cartilage (i.e. less GAG and $^{35}SO_4$-labeled GAG in stimulated samples compared to non-stimulated samples), the model applied in this study could be considered efficient in producing a catabolic condition in articular cartilage.

Because most of the studies that used similar models as the ones applied in this experiment used FBS as media supplementation, FBS was used in control samples as well as the ITS to validate the efficiency of the model in comparison with other studies[17]. Here, cartilage samples responded similarly to IL-1β stimulation despite the type of media used, further supporting the model implemented.

The effect of PL in cartilage under a pro-inflammatory environment was assessed in this study (Analysis 2). PL treated samples were significantly different compared to the ITS+IL-1β control group for total GAG (media and cartilage) and $^{35}SO_4$-labeled GAG in cartilage. In a model such as applied in this study, matrix degradation induced by IL-1β insult leads to increase in proteoglycan loss to the media and therefore the $^{35}SO_4$-labeled GAG left in cartilage can be accessed. Consequently, less $^{35}SO_4$-labeled GAG in cartilage indicates more degradation of newly synthetized GAG. Hence, similar content of $^{35}SO_4$-labeled GAG retained in cartilage explants at the termination of the study for PL treated samples and controls suggest that all groups had similar degradation of newly synthetized GAG during the experiment. Such findings indicate that PL have failed in protecting cartilage against the catabolic effects of IL-1β. These results are not in accordance with a series of studies showing the anti-inflammatory and anabolic effects of PRP in different in vitro culture models [27] [28]. In Osterman et al[27] for instance, a co-

culture system with osteoarthritic cartilage and synovium was exposed to IL-1β and treated with two PRP preparations (high leukocyte-platelet PRP and low leukocyte-platelet PRP). Both PRP preparations were significantly anti-inflammatory, demonstrating to decrease the expression of catabolic factors evaluated [27]. Furthermore, in vivo the use of PRP showed significant clinical and functional improvement in OA patients up to 6 months after treatment compared to control [29]. Thus, the findings demonstrate that PRP has a potential anti-inflammatory and protective effects that were stated previously, but that were not observed in this study.

The lack of significance in total GAG (Figure 5) and $^{35}SO_4$-labeled GAG content (Figure 6) observed between PL treated samples and control might be explained by factors related to the PL used in this study. It is well known that PRP effects are influenced by its cellular content and protocol of activation.

Although freeze-thawing was previously used as a form of platelet activation [30], due to partial lyses of the platelets this method could have resulted in a final product with lower content of certain growth factors compared to more traditional methods of activation [31]. This could justify the lack of significance between frozen PL and control in this study. Nevertheless, for the freeze-dried and filtered freeze-dried PL, the rehydration of the platelets would most likely lead to membrane destabilization and rupture releasing platelet's content [32]. Still, once no agents for membrane and protein stabilization were used before lyophilization (as done in previous studies [30]), it's possible that the lyophilization process could have partially damaged the PL's cytokine content [33]. Therefore, both freeze-thaw and lyophilization process used in PL might have resulted in poorer cytokine content compared to other forms of PRP activation, which could explain the non-protective results of PL in this study.

The effect of treatment was not significant for the biochemical analysis in this study (Analysis 3). Freeze-dried PL preparations demonstrated to be have equivalent effect compared to Frozen in cartilage matrix. These results agree with previous findings comparing the use of freeze-dried and fresh PRP. Specifically, freeze-dried platelets preparations demonstrated similar increase in granulation, proliferation and angiogeneic response compared to fresh/frozen PRP in a diabetic wound mouse model [34].· Such results provided proof-of-principle that freeze-dried formulations of PRP can potentially be equivalent to frozen preparations.

Although no significant difference was observed between Frozen and Freeze-dried PL for the biochemical analysis, differences between these treatments were observed in gene expression. IL-1β is known to induce upregulation of a series of cytokines and proteolytic enzymes in chondrocytes in an inflammatory condition [35, 36]. Thus, to access the potential modulatory effects of PL in the downstream cytokines induced by IL-1 β, gene expression of ADAMTS-5, ADAMTS-4, MMP-1, COX-2 and IL-1β were assessed in cartilage.

ADAMTS-4 and 5 are upregulated in injured cartilage and joints with OA [37, 38] inducing cartilage degradation. As with other collagenases, increase expression of MMP-1 has been associated with cartilage degradation, and it can be up-regulated in cartilage for longer periods of culture in vitro compare to aggrecanases [25]. IL-1 is a major cytokine in OA disease triggering the increase of innumerous enzymes such as COX-2, propagating the inflammation within the joint [39]. In this study, all the genes assessed were upregulated with the use of PL and the upregulation in gene expression demonstrated to be variable depending upon the PL preparation used. The increase in expression levels of catabolic factors indicate that the PL used in this study was potentially pro-inflammatory for cartilage in the model adopted.

Deleterious effects of leukocyte-rich PRP has been demonstrated in tissue culture [40]. Although the PL used in this study had leukocyte counts bellow the baseline for the whole blood for equine, the freeze-thawing and lyophilization of PL could led to rupture of the white blood cells (and possibly red blood cells) present within it. Therefore, the rupture of these cells and consequent release of cytokines as well as the cellular debris could have potentially caused a pro-inflammatory effect in cartilage during this experiment.

To date, only one study compared the differences in gene expression between fresh PRP preparations and lyophilized platelets in tissue culture. McCarrel and Fortier (2009) [32] evaluated the effects of bone marrow and different PRP formulations in tendon and ligament explants in vitro. The authors observed that both fresh and freeze-dried PRP preparations had anabolic effects. Both formulations increased gene expression of collagen1 A1: collagen3 A1 ratio and cartilage oligomeric matrix protein (COMP) and decrease MMP-13 expression in tendon and ligaments explants. Nonetheless, the authors only verified PRP effects in normal explants, thus the outcomes of such formulations under inflammatory conditions could be potentially different.

Furthermore, it's important to consider that studies in this specific area are scarce and no studies at this point have compared the effects of fresh/frozen and lyophilized platelets in cartilage or in tissue culture under inflammatory conditions. Therefore, the effects of PRP formulation can vary considerably depending upon the tissue and condition in which the product is tested, which could justify the different results presented here. Besides, previous studies that claimed similar growth factor content in freeze-dried platelet products compared to fresh/frozen preparations [30] [41] did not do a systematically analysis of the cytokine content of these products. Therefore, many biological factors that could potentially explain the differences obtained in this study have yet to be explored. Nonetheless, freeze-drying PL affected the gene-expression of

pro-inflammatory enzymes differently compared to frozen preparations, and those effects should be considered for future studies.

Overall, autologous and allogeneic PL demonstrated to be equivalent in this study. These findings suggest comparable effect of Autologous and Allogeneic PL in cartilage matrix. Similar results were obtained comparing Autologous and Allogeneic forms of PRP and PPP in fibroblasts in vitro, where both forms tested presented equivalent results for cell migration and proliferation [42]. Moreover, in a large bone defect in vivo model, Allogeneic PRP demonstrated to be efficient in enhancing bone healing. Besides it provided a potentially more consistent quality compared to autologous preparations [43]. Considering the findings obtained in this study, the allogeneic and autologous PL demonstrated equivalent effect in cartilage matrix.

For relative gene expression however, the autologous PL demonstrated to be significantly more upregulated compared to allogeneic PL for expression of IL-1β. However, this was not observed for the other genes evaluated in this study, and both conditions lead to upregulation of catabolic factors. No similar findings were observed in previous reports. Nevertheless, once this study was performed in vitro, it did not account for the effects of the allogeneic preparations in other tissues within the joint (such as synovium and subchondral-bone) that are known to play a paramount role in osteoarthritis development and progression. Therefore, further investigations in other tissues in vitro and vivo are warranted to fully understand the effects of allogenic PL.

The main effect of Concentration demonstrated significant effect in the biochemical analysis in cartilage (Analysis 3). The concentration of PL did seem to demonstrate significant effect in cartilage matrix and the 3x PL concentration had significantly more $^{35}SO_4$-labeled GAG, compared to both 1.5 x and 6 x concentrations. $^{35}SO_4$ was used to label the GAG in cartilage explants before the commencement of the experiment. Therefore, once GAG was loss

to the media due to catabolism induced by IL-1β, higher levels of $^{35}SO_4$ -labeled GAG in cartilage for the 3 x PL groups indicate less degradation compared to the 1.5 x and 6x concentrations. This suggests that the dose-response curve for PRP effects is not linear in cartilage matrix [44]. Thus, the results presented here agree with previous findings in which increase of platelet concentration does not necessarily improve PRP results. For instance, different concentrations of PRP (3x, 6x and 9x compared to baseline) induced similar TGF-β release into the media and radioisotope incorporation in matrix in a cartilage explant model [45]. Consequently, the authors concluded that increase in concentration of platelets in PRP does not stimulate cartilage matrix synthesis [45]. While an excessive increase in platelet concentrations can lead to inhibitory effects [44], lower concentrations might not provide enough anabolic stimulation [46]. Thus, intermediate concentrations might be more appropriate for PRP use [46].

The upregulation in gene expression of catabolic factors suggests a pro-inflammatory effect of PL in cartilage. PL cellular content, method of activation (freeze-thaw) and the lyophilization process implemented in this study could have affected the cytokine profile and kinetics of release leading to these results. However, the expression of pro-inflammatory factors induced by PL are in contradiction with what was observed in the biochemical analysis performed. In cartilage matrix, PL did not induce significant changes compared to the ITS+IL-1β control group. The gene expression induced by PL could have changed over time during culture and consequently the changes in matrix may not be detectable at the time of sample collection. In addition, PL could have interfered with other cytokines not investigated in this study or with other mechanisms not at the gene expression level that could have interfered with cartilage matrix homeostasis.

In sum, the multiple cytokines present within PL could have played a role in the anabolic and catabolic balance in cartilage matrix that could explain the discrepancies observed between cartilage matrix data and gene expression.

5. Conclusion

In this experiment, the PL used failed in protecting cartilage from the inflammatory stimulation of IL-1β, and in fact demonstrated to be potentially pro-inflammatory inducing up-regulation of the inflammatory factors tested. Yet, such changes were observed only in gene expression and not in cartilage matrix.

The effects of different concentrations of platelets in PL had a significant effect in this experiment, and intermediate levels of platelets demonstrated to promote more beneficial effects compared to higher and lower platelets content, and therefore might be a better option when applying PRP therapeutically.

The different formulations of PL used in this study (frozen versus freeze-dried and allogeneic versus autologous) had equivalent effect in cartilage matrix changes; however, were significantly different for gene expression of the catabolic factors evaluated, which were variable depending on the PL formulation used.

Therefore, the hypotheses 1 and 2 of the study were rejected. Freeze-dried PL did not demonstrate to be equivalent to frozen in gene expression (hypothesis 1), and allogeneic PL did not demonstrate to be equivalent to autologous condition (hypothesis 2) in gene expression. Finally, the formulations of PL investigated in this study did demonstrated different modulatory effects in cartilage in the model used, but that overall did not seem to interfere with the cartilage matrix metabolism. Further investigation of the allogeneic freeze-dried PL effects in synovium in vitro as well in in vivo models of OA are indicated.

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CHAPTER 3

Evaluation of allogeneic freeze-dried conditioned serum for the prevention of pathologic changes in joint tissues exposed to inflammatory conditions

1. Introduction

Osteoarthritis (OA) is one of the most important causes of equine and human musculoskeletal disability. The complete pathogenesis is still poorly understood, but loss of anabolic and catabolic homeostasis contributes to the progressive degeneration characteristic of OA [1]. In the past few decades, more interest has been given to drugs that could modulate OA development. Those areas of interest include the inhibition of target cytokines involved in inflammatory pathways in this disease [2]. Although IL-1 is not the only pro-inflammatory protein enrolled in OA [1] pathogeneses, studies suggest its action is the most promising approach for cytokine blocking [3]. IL-l receptor antagonist (IL-1Ra) is a naturally occurring protein belonging to interleukin-1 (IL-1) family and competes for occupancy of the IL-1 cell surface receptors, but doesn't initiate catabolic response typical of IL-1 [4]. Thus IL-1Ra is a natural inhibitor of IL-1. The efficacy of IL-1Ra delivery in vivo has been previously showed through the use of gene therapy [5]. However, simpler forms for delivery of high levels of IL-1Ra has been developed, and one example of it is autologous conditions serum (ACS). Autologous conditioned serum (ACS) is produced following culture of whole blood in the presence of medical grade coated glass beads. This process results in serum that is enriched in endogenous IL-1Ra as well as anti-inflammatory cytokines (such as IL-4, IL-10) and growth factors, with little induction of pro-inflammatory cytokines [6].

ACS has also been tested in vivo and demonstrated significant improvement in clinical lameness and decreased pathologic changes in osteoarthritis affected joints in horses [7]. While positive clinical results have been obtained with the clinical use of commercial ACS products, multiple limitations preclude its widespread acceptance. One limitation is the inconvenience in preparation of ACS for most equine practitioners. Specifically, in most circumstances when ACS is used clinically the practitioner must submit the blood to multiple steps. After drawing, the blood is taken back to the practice and incubated using a commercial kit for 24 hours to harvest the ACS. Followed by centrifugation, the ACS is then frozen in multiple aliquots and then applied into the patient on three separate occasions. To preserve its integrity, the ACS must be kept frozen at -20°C until its use, which is not always possible in many clinics and on the field. Besides those factors, ACS presented significant variation in both pro and anti-inflammatory cytokine based on the patient.

Based on those issues, this study proposed to create an allogeneic freeze-dried conditioned serum (CS). The lyophilized product would be stable at room temperature allowing easy storage and transport and by pooling blood from different horses (allogeneic treatment) a more homogeneous and quantified product would be created. This study is based on the following hypotheses:1) Freeze-dried CS will have similar effects compared to frozen preparations in cartilage and synovium stimulated with IL-1β, 2) Allogeneic CS will have similar effects compared to autologous preparations in cartilage and synovium stimulated with IL-1β and 3) CS effects will be concentration dependent in cartilage and synovium stimulated with IL-1β. The hypotheses were tested in an in vitro explant laboratory model using tissues harvest from mature horses.

2. Material and Methods

All horses utilized for the experiment were mature and healthy (2 -5 years of age) and were part of an unrelated terminal study, thus no increase animal wastage occurred. Blood was collected from these horses and cartilage or synovium explants were harvest from the same horses after study termination and euthanasia. Eight horses were utilized for the cartilage experiment and eight different horses used for the synovial experiment. The protocol for blood collection was approved by the Animal Care and Use Committee of Colorado State University (ACUC, protocol number: 14-5477A for the cartilage section, and 16-6377 for the synovium section).

2.1 ACS production

2.1.1 Bead processing

3 mm borosilicate glass beads were used for blood incubation and production of the ACS in this experiment. The beads used in this experiment were processed according to a method previously optimized by our laboratory that yielded similar IL-1Ra levels compared to ACS commercial kits [8]. Subsequently to production, beads were weighed 11.824g and place into 50 mL conical tubes. All the tubes and the beads were sterilized using Ethylene Oxide before use.

2.1.2 Blood processing and incubation

Blood was collected from each horse separately and aliquoted into 50 ml conical tubes containing coated beads for ACS preparation. Blood was incubated for 18-24 hours in a 37°C incubator. Subsequently incubation, the tubes containing beads and the blood were centrifuged for 10 minutes at 4000 rpm (1716.88 xg) in a Hermile ZZ300-Labnet centrifuge. Serum was then removed from the tube, and care was taken to not disturb the clot in the bottom of the tube. Serum was aliquoted in smaller volumes and stored at -80°C freezer until the experiment was

initiated. To create the freeze-dried and frozen treatments, the ACS aliquots from each horse was divided in two equal parts, one part remained frozen at -80°C until the experiment was initiated (frozen treatment) and the other was lyophilized for 18 hours and then returned to -80°C until the commencement of the experiment (freeze-dried treatment). The filtered freeze-dried treatment was processed similarly to the freeze-dried group however, the serum was filtered with a 45µm syringe filter before lyophilizing. This group had the objective to mimic the procedure done clinically (where ACS is sterile-filtered before application), and to verify if filtrating serum could impact its effect. For the autologous CS, the ACS produced were kept separated to be applied into the explants of the same horse the blood was collected (Figure 3.1).

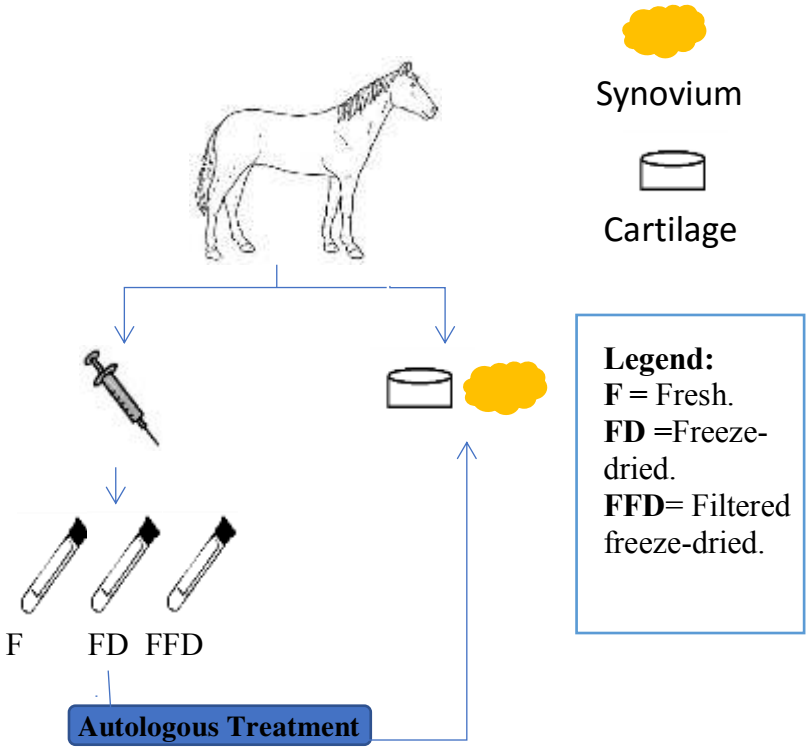

Figure 3. 1: Autologous CS. Blood was collected from eight horses for each section of the experiment (N=8 for cartilage and N=8 for synovium) and processed to produce the ACS. All the ACS were processed in three different treatments; frozen, freeze- dried and filtered freeze-dried. The ACS produced was then applied into the cartilage or synovium explants from the same horse the blood was collected. The application of ACS was done in two different concentrations (10% and 30%).

For the allogeneic CS, the aliquots from four horses were pooled together (Figure 3.2). The effect of autologous and allogeneic CS in this study was indicated as Allogenicity. The mentioned Treatments and allogenicity were applied in two different Concentrations, 10% and 30% in media (volume/volume).

2.2 Cartilage explant harvest

Immediately after euthanasia, cartilage from the stifle joint was harvested from each horse, using an 8mm punch to obtain samples with wet weights (ww) between 70-100 mg per explant. Care was taken not to harvest tissue below the calcified cartilage layer. The explants were placed into a 24 well plate in 1 mL of culture medium [Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 0.1 mM nonessential amino acids, 10 mM HEPES, 0.4 mM proline, 0.16 mM of α-ketoglutaric acid, 1 mM of sodium pyruvate, 2 mM of L-alanyl-L-glutamine (Glutamax, Gibco), penicillin (100 U/mL) and streptomycin (100 μg/mL)] [9]. The media used for plating the explants (and during the role experiment) was supplemented with either fetal bovine serum (FBS), or insulin transferrin-selenium (ITS) as media supplement.

FBS is widely used in tissue culture, however the presence of cytokines and growth factors could potentially interfere with the components of the conditioned serum tested in this study [10]. For this reason, samples that were treated with CS had serum-free media, supplemented with ITS [11], instead of FBS.

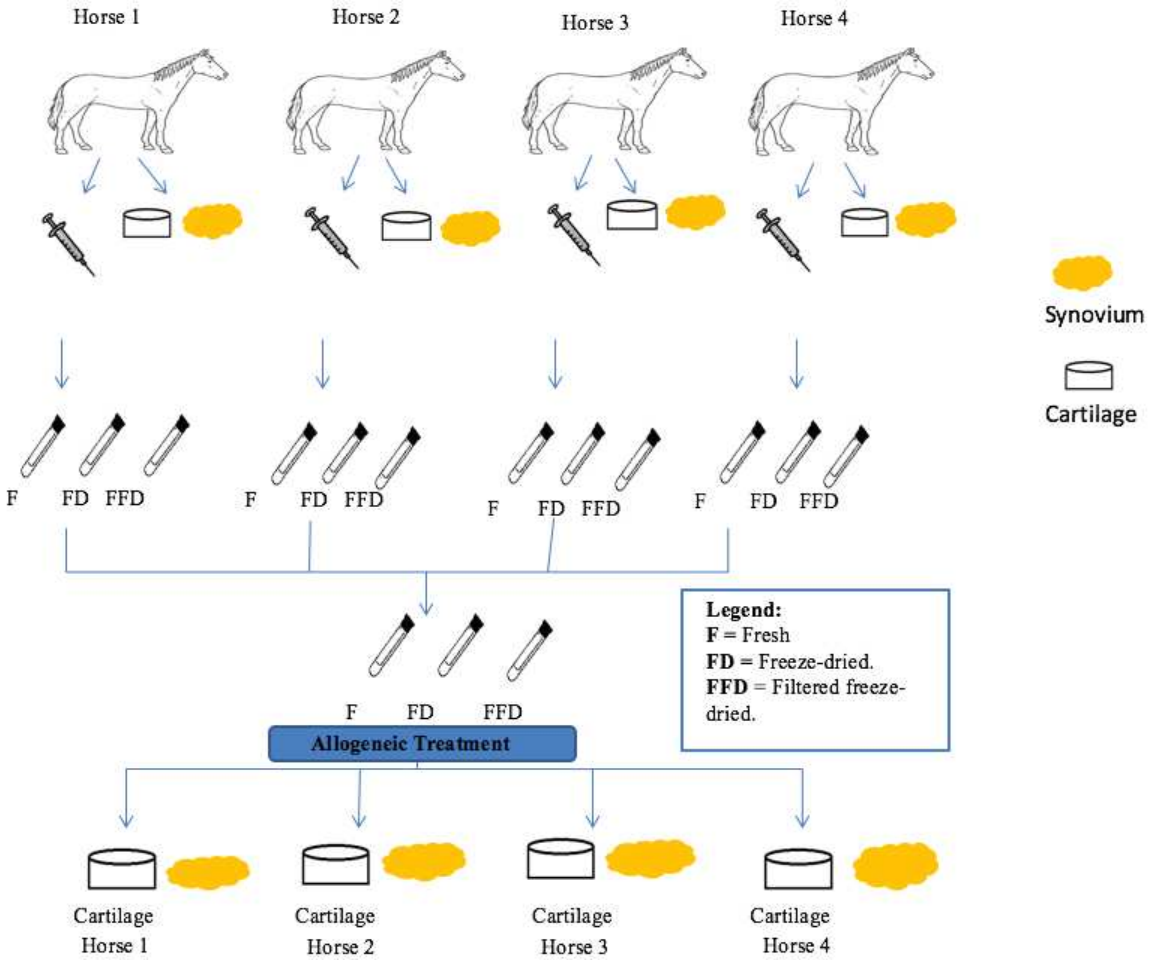

Figure 3. 2: Allogeneic CS. Blood was collected from eight horses for each section of the experiment (N=8 for cartilage and N=8 for synovium) and processed to produce the ACS. All the ACS were processed in three different treatments; frozen, freeze- dried and filtered freeze-dried. Before use, the ACS from each horse of a group of 4 horses (2 groups N=4) was combined before being applied to the explants. The application of the allogeneic CS was done in two different concentrations (10% and 30%).

Yet, to validate the efficiency of the model in this experiment and compare to previously studies, FBS was used for supplementation of control samples as well as ITS.

Cartilage samples that were submitted to CS treatments or were serum-free media controls received media with 1 % ITS Premix volume/volume (6.25 µg/mL of insulin, 6.25 µg /mL of transferrin and 6.25 ng/mL of selenium acid, 5.35 mg/mL of linoleic acid and 1.25 mg/mL of Bovine Serum Albumin -BD Bioscience). Cartilage explants that were FBS control samples received media with 10% FBS. After plated in fresh medium with the proper media supplementation, samples were left to equilibrate for 48 hours in a humidified incubator at 37°C.

2.3 Synovium harvest

Synovial membrane was collected immediately after euthanasia, from both the metacarpal-phalangeal joint and the carpal joints. Samples were then randomly assigned for the different groups of the experiment. The synovial tissue was cut into explants (approximately 5 mm x3 mm) [12] and placed into culture 24 well culture plates in F12 growth medium [Ham's F12 (Invitrogen, Carlsbad-CA), 20% Fetal bovine serum (Invitrogen, Carsbad-CA) , 0.2% antibiotic v/v (Invitrogen, Carlsbad-CA), 10 mM HEPES (Invitrogen, Carlsbad-CA), 0.28 mM of ascorbic acid and 0.16 mM of α-ketoglutaric] and let to equilibrate for 48 hours.

Before the initiation of the experiment, the FBS media was replaced by media supplemented with 1% ITS +Premix (25 µg/mL of insulin, 6.25 µg /mL of transferrin and 6.25 ng/mL of selenium acid, 5.35 mg/mL of linoleic acid and 1.25 mg/mL of Bovine Serum Albumin, ITS + Premix Universal Culture Supplement, Corning), for samples that would be treated with CS or were ITS controls. FBS control samples remained in FBS media for the rest of the experiment.

2.4 Explant culture

Following preparation and storage, each aliquot of CS treatment was thawed, rehydrated in media (freeze-dried and filtered freeze-dried CS aliquots) and subsequently applied at a 10% or 30% concentration (volume/volume) into culture wells containing explants (synovium or cartilage). Autologous CS were applied directly to the explants from the same horse the blood was collected. Allogeneic condition had aliquots from different horses combined to create the allogeneic CS, that was then applied to explants. For each Treatment, Concentration and for autologous and allogeneic conditions (Allogenicity), triplicate samples were used. Regarding to the controls each control group (ITS or FBS) were exposed to IL-1β stimulation (ITS+ IL-1β and FBS+ IL-1β) or not (ITS or FBS controls). The main effects of IL-1β and media supplementation used (ITS or FBS) are designated in this experiment as IL-1β and MEDIA, respectively. A summary of the different CS and controls is described on Figure 3.3.

At the commencement of the experiment (Day 0) the explants were stimulated to both IL-1ß [10 ng/mL [13] diluted in 0.1% bovine serum albumin (BSA, Sigma Chemicals) and PBS] and the appropriate CS, as described. Explants received a second dose of IL-1ß and CS on Day 4 of the experiment. At all other days, the explants received culture medium only. Medium was changed and collected at 2 day intervals and stored at -80°C for later analysis. On the 10th Day of the experiment the explants and the media were collected and frozen in -80°C until analysis (Figure 3.4).

2.5 Biochemical analysis

2.5.1 Cartilage explant digestion

Cartilage explants were digested for biochemical analysis. Explants were weighed, lyophilized and dry weights were recorded. Samples were papain digested with a crystallized papain solution [14] overnight at 60°C. After digestion, samples were kept at -80°C until analysis.

2.5.2 Explant DNA quantification

DNA content was determined using a fluorescent dye based assay (Hoechst 33258) [15]. In brief, digested explants were added to a diluted dye/buffer solution, placed in duplicates and read against a standard curve using calf thymus DNA (Sigma) on a plate reader (Molecular Devices Spectramax M3). Hoechst dye interacts with DNA in the digested samples resulting in a change in color which is analyzed through spectrophotometry. In this experiment, the values were measured using a fluorometer with excitation of 360 nm and emission reading at 460 nm. DNA content was evaluated to be used for normalization of the biochemical data in cartilage explants.

2.5.3 Explant glycosaminoglycan (GAG) quantification

To evaluate the effects of CS in cartilage matrix under inflammatory conditions, total GAG content in cartilage and media were accessed using a modified method of the DMMB assay [16]. In brief, the samples were added to a dye/buffer solution and read against a standard curve of Chondroitin Sulfate C from shark cartilage (Sigma). The DMMB dye form complexes when bound to sulfated glycosaminoglycan, which results in color change within the sample, which is measured using spectrophotometry. In this experiment samples were read at 530nm in a plate reader (Molecular Devices Spectramax M3).

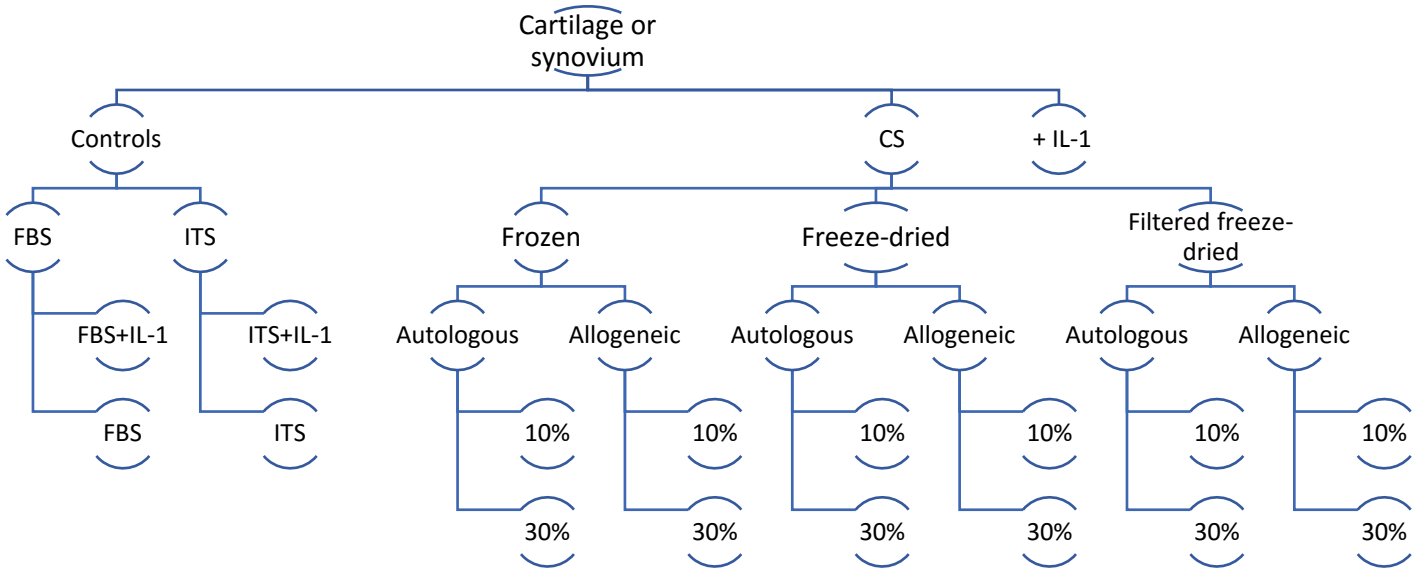

Figure 3.3: Diagram of the different CS groups and controls used in the study for cartilage and synovium experiments.

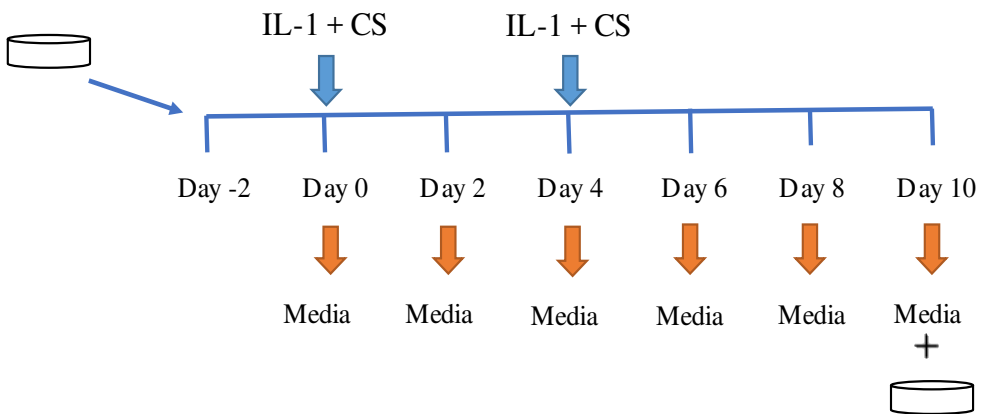

Figure 3.4: Study timeline. Samples were collected and left in complete media to equilibrate for 48 hours before the commencement of the experiment. At Day 0 and Day 4, samples were exposed to IL-1β and different CS treatments, concentrations and in autologous or allogeneic conditions. In all the other Days of the experiment, samples received full growth media only.

The change in color intensity is correlated to the amount of glycosaminoglycan found in the sample. All samples and standards were run in triplicate. GAG data was normalized to DNA content in cartilage and presented as µg of GAG/ µg of DNA.

2.5.4 Explant $^{35}SO_4$-labeled proteoglycans quantification

$^{35}SO_4$ isotope incorporated into proteoglycan ($^{35}SO_4$ - labeled GAG) was evaluated in the cartilage explants by the end of the experiment, as well as what was released into the media during the experiment. The $^{35}SO_4$ - labeled GAG evaluated at the termination of the study in cartilage and media was used as an indirect measure of newly synthetized GAG degradation.

Sixteen hours prior to the commencement of the experiment, 15 µCi of $^{35}SO_4$ was added to each well. After incubation and before treatment, samples were washed with fresh media and exposed to IL-1β stimulation and treatments (Day 0). Upon completion of the experiment (Day 10), the concentration of $^{35}SO_4$ incorporated in the explant matrix and released into the media was measured using a modified Alcian blue system. Briefly, samples were added in duplicate to

a 96 Multiscreen well plate, along with assay buffer and Alcian blue dye. The multiscreen plates were attached to a vacuum manifold which draw the solutions through the screen allowing only the radiolabel bound to GAG and Alcian blue to be catch in the screen. The screen was then analyzed via liquid scintillation counting. For standards, solutions containing different concentrations of $^{35}SO_4$ were also analyzed via liquid scintillation counting [17]. $^{35}SO_4$-labeled GAG was normalized to DNA content in explants and presented in DPM/μg of DNA.

2.6 Gene Expression

To verify the potential modulatory effect of CS in cartilage and synovium under inflammatory conditions, gene expression of catabolic enzymes and cytokines related to OA were accessed.

2.6.1 Cartilage RNA extraction

After experiment termination, cartilage explants were collected from culture, weighted and immediately placed in Tryzol reagent (Invitrogen) and stored at -80°C until analysis. To perform the RNA extraction, samples were thawed and placed into microcentrifuge tubes for pulverization for 30 seconds using a BioSpec 31100B X Mini-BeadBeater-1. Immediately after pulverization, Tryzol was added and samples were homogenized, incubated at room temperature and then centrifuged for 10 minutes at 12,000 xg. Supernatant was collected and nucleic acids were extracted with the addition of 20% chloroform (volume/volume) and centrifuged for 15 minutes, at 12,000 xg. If necessary, this step was repeated multiple times to ensure separation of nucleic acids from extra-cellular matrix. Nuclei acids were then precipitated with isopropanol and centrifuged for 10 minutes at 10,000 xg. All centrifugations were performed at 4°C. After the last centrifugation, the nucleic acid pellets were then resuspended in 30 μL of nuclease-free water.

2.6.2 Synovium RNA extraction

Synovium explants were collected after study termination and placed into microcentrifuge tubes containing Buffer RLT (Qiagen). Samples were placed immediately at -80°C until analysis. To extract RNA from synovium explants, RNeasy Mini Kit (Qiagen) was used. Briefly, after disruption of the synovium explants using the 31100B X Mini-BeadBeater-1, Buffer RLT was added and sample was pipetted multiple times assuring the disruption of the tissue. One volume of 70% ethanol was added to the lysate and homogenized. After homogenization, lysate was transferred to RNeasy spin column, placed in a collection tub and centrifuged for 15 seconds at 8000 xg. After multiples washes of the column with buffers, RNA was eluted using nuclease-free water. Nucleic acid yield and purity from both cartilage and synovium were determined at 260/280 nm using a Nanodrop ND-1000 Spectrophotometer.

2.6.3 Reverse-transcription

After extraction, RNA was treated for genomic DNA contamination and reverse-transcribed simultaneously using a blend of oligodT and random primers (iSript^{TM}gDNA Clear cDNA synthesis kit, Bio-Rad). DNase treatment was done for 5 minutes at 25°C and DNase inactivation was done for 5 minutes at 95°C. Reverse-transcription protocol was performed as follow: priming for 5 minutes at 25°C, reverse-transcription for 20 minutes at 46°C and enzyme inactivation for 1 minute at 95°C. cDNA was stored at -80°C until use.

2.6.4 Quantitative Polymerase Chain Reaction (qPCR)

cDNA solution and iTap^{TM}Universal Syber ®Green Supermix (Bio-Rad) were mixed with primers for the genes of interest and gene expression levels were determined by quantitative real-time PCR using a CFX96 Touch^{TM}Real-Time PCR Detection System (Bio-Rad). Conditions were: initial activation step 3 minutes at 95°C, denaturation 10 seconds at 95°C, annealing 30

seconds at 60°C and melting temperature 65°C-95°C, incrementing 0.5°C for 0.5 second. Expression levels of IL-1ß, matrix metallopeptidase-1 (MMP-1), a desintegrin metalloproteinase with thrombospondin motifs 4 (ADAMTS-4), ADAMTS-5, IL-1ß, COX-2 and ubiquitin C (UBC) were evaluated for cartilage samples. Expression levels of IL-1ß, COX-2 and UBC were analyzed in synovium. All primers were designed using PrimerQuest Tool (Integrated DNA Technologies - IDT) and National Center of Biological Information (NCBI) software. Characteristics used for primer designed were; size under 150 nucleotides, primer should span in an exon-exon junction, having CG% approximately in 50%, melting temperature close to 60°C and the minimum of hairpins or secondary structures. Efficiencies curves were performed using 10-fold dilutions of cDNA from equine chondrocytes and synoviocytes exposed to IL-1β. When the efficiencies were bellow 90%, PCR products were run in a 2% agarose gel as a confirmation that the target sequence was amplified. Primer sequences are shown in Table 3.1.

Gene expression levels of the target genes were normalized to the ubiquitin C (UBC) as endogenous control (Δ_{CT}). The Δ_{CT} of the target samples were then subtracted from the Δ_{CT} of the calibrator resulting in the $\Delta\Delta_{CT.}$ The calibrator used in this experiment was the control samples supplemented with ITS in media and exposed to IL-1β (ITS+IL-1 β). The gene expression was presented in fold ($2^{-\Delta\Delta CT}$) change relative to the ITS+IL-1 β control. For the synovium experiment, FBS+IL-1β control were used as a calibrator. Since a Ct of 38.5 was still within a range for the efficiency curves of a few primers developed for this study, samples with a Ct above 38.5 were not considered for analysis.

Table 3.1: Sequences of primers used in the study.

Primers used in the study	**Primer sequence (5' to 3')**
ADAMTS-5	Forward: AAGGTGACTGATGGGACCGAATGT

	Reverse: TTTGAGCCAATGATGCCGTCA
ADAMTS-4	Forward: GTCCCAAAGGCCACATACTT
	Reverse: GCCACTCTTACTTGCCCATATC
MMP-1	Forward: ACTGCCAAATGGACTTCAAGCTGC
	Reverse: TCTTCACAGTGCTAGGAAAGCCG
COX-2	Forward: GCACCATAGATGCTCAAGAAGTA
	Reverse: GACTGTGAGGGACGGATTATTG
IL-1β	Forward: CCAGAGGCGGCCGGGACATAAC
	Reverse: GGGAAGGCAGCTGGGCATTGATT
UBC	Forward: GGCTGTTAGCTTTTCAGTCTTGTC
	Reverse: CTTAAATTGGGGCTAATGGCTGG

2.7 Statistical analysis

Three different analyses were performed in this study: 1) To evaluate the effect of IL-1β stimulation. 2) To evaluate the effect of CS treatments compared to ITS control media both in a IL-1β stimulated environment. 3) To evaluate the effects of Treatment, Allogenicity and Concentration in CS treated samples in a IL-1β stimulated environment.

For all analyses a mixed-model of variance using the GLIMMIX procedure in SAS 9.3 (SAS Institute) was used. Media GAG and $^{35}SO_4$-labeled GAG, Explant GAG and $^{35}SO_4$-labeled GAG were all considered as dependent variables for all three analyses. Gene expression was considered as a dependent variable for Analysis 3. Independent variables are described according to the analysis performed. Horse was defined as a random effect in this study.

For analysis 1: the independent variables were IL-1β and type of media (MEDIA). Only control samples were evaluated in this analysis. The main effects of IL-1β and MEDIA as well as the interactions were considered.

For analysis 2: the independent variable was Treatment. CS treated samples were compared to ITS+IL-1β controls. Only the main effect of Treatment was considered in this analysis.

For analysis 3: the independent variables were Treatment, Allogenicity and Concentration. In this analysis only CS treated samples were considered. The main effects of Treatment, Allogenicity and Concentration as well as interactions were evaluated.

The estimation technique in the mixed model used was restricted maximum likelihood (REML). Normal distribution was accessed for depend variables using student residual plots. If restricted maximum likelihood (REML) was not adequate and normality assumption was not met, log transformation was performed. Outliers were identified based in diagnostic residual plots as well as any values above a mean ± 3 x standard deviation. Outliers were then excluded from the data set and statistical analysis was performed once again. To account for multiple comparisons errors, a protective F-test was done for the main effects and interactions for all three analysis. Then, least square means was used for the individual comparisons. A P value < 0.05 was considered significant. When reporting significant results for the main effect or interactions the highest order is reported, further only significant outcomes are reported. Values reported are means ± standard error of the mean (mean ±SEM). In the figures reported bellow, bars represent the standard error of the mean and different letters indicate a significant difference between groups.

3. Results

3.1 Evaluation of IL-1β stimulation in cartilage (analysis 1)

This analysis was performed to assess IL-1 stimulation of the cartilage explants. The outcome parameters collected were total GAG and $^{35}SO_4$-labeled GAG in media and explant utilizing the main effect variables of IL-1β and MEDIA and well as IL-1β*MEDIA interactions.

3.1.1 Media GAG

The main effect of IL-1β was statistically significant for Days 2, 4 and 6 (F test, P < 0.005) independent of the type of media used (Figure 3.5).

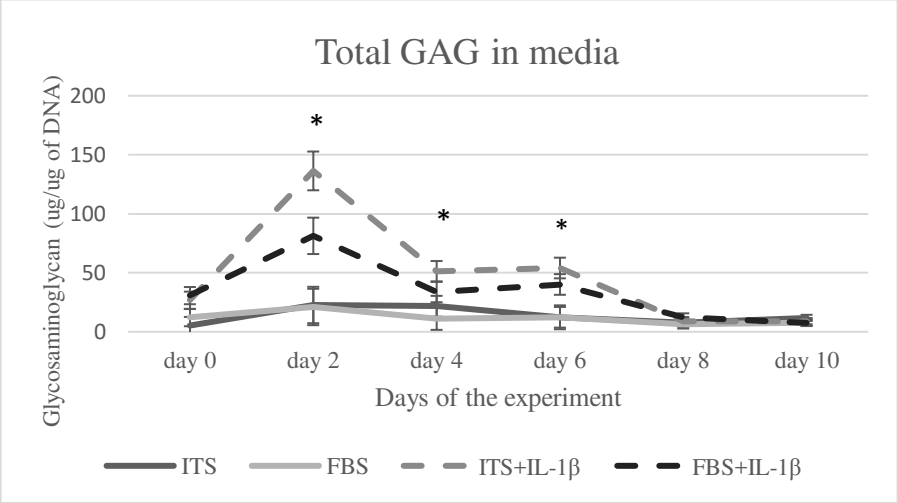

Figure 3.5: Total GAG in the media during the experiment for control samples. Control samples stimulated with IL-1β loss significantly more GAG to the media on days 2, 4 and 6 of the experiment compared to samples non-stimulated with IL-1β (in both ITS and FBS supplemented controls), illustrated by the "*" symbol. Level of significance used 0.05.

3.1.2 Total GAG in cartilage

Presence of IL-1β resulted in significant GAG loss in cartilage explants at the end of the study (F test, P= 0.0143, Figure 3.6). ITS+IL-1 β, FBS and FBS+IL-1β had significantly more GAG loss compared to ITS control samples (Figure 3.6).

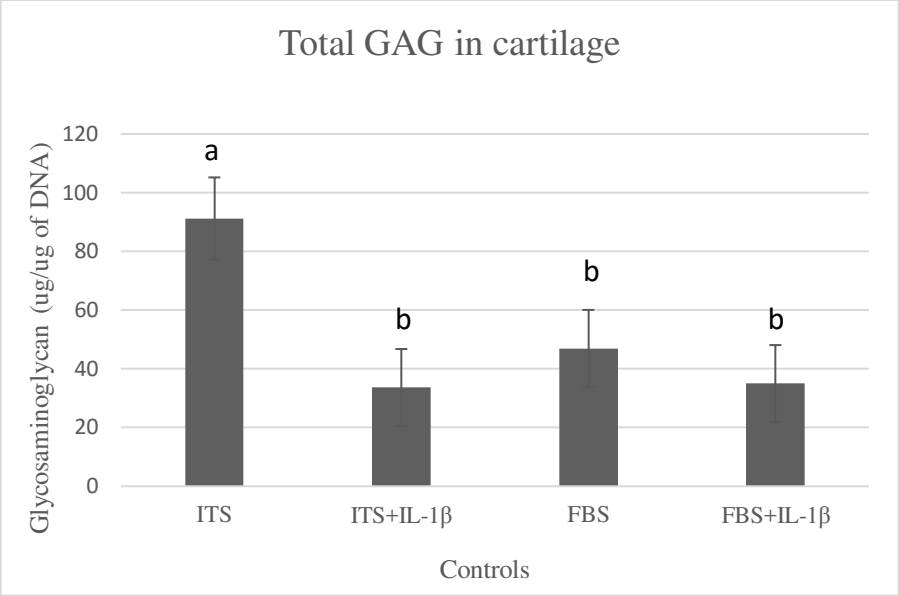

Figure 3.6: Total GAG in cartilage for control samples. ITS+IL-1β, FBS and FBS+IL-1β controls had significantly more GAG loss compared to ITS controls. Level of significance used 0.05.

3.1.3 $^{35}SO_4$-labeled GAG in media

A significant effect of IL-1β was observed for $^{35}SO_4$-labeled GAG in media at Day 2 and 4 (F test, $P<0.0339$) of the experiment (Figure 3.7). The main effect of MEDIA was significant at Days 4 and 8 (F test, $P<0.025$). The interaction between media MEDIA*L-1β was as well significant at Day 2 and 6 (F test, $P<0.0347$. Figure 3.7). At Day 2, ITS+IL-1β control had significantly more $^{35}SO_4$-labeled GAG in media compared to FBS+IL-1β and ITS controls (Figure 3.7). At Day 4, ITS+IL-β had significantly more $^{35}SO_4$-labeled GAG compared to ITS, FBS and FBS+IL-1β (Figure 3.7). Finally, at Day 8 ITS+IL-1 had significantly more $^{35}SO_4$-labeled GAG in media compared to FBS control (Figure 3.7).

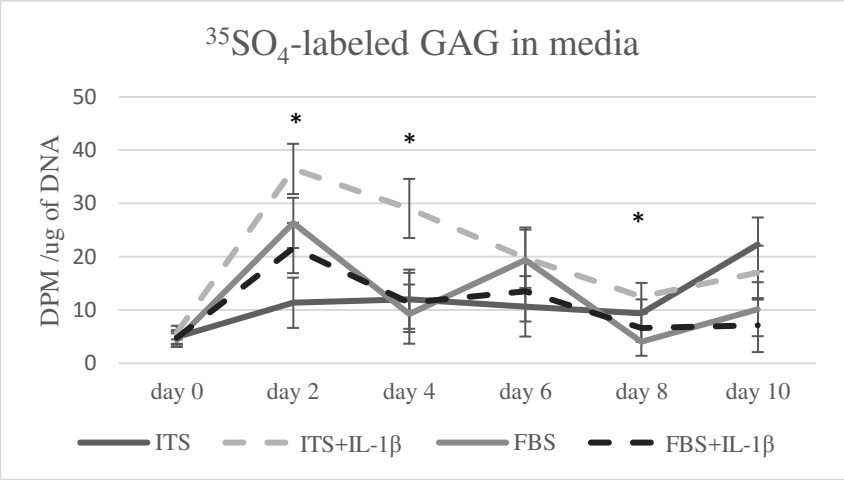

Figure 3.7: $^{35}SO_4$-labeled GAG in media during the experiment for control samples. Samples exposed to IL-1β lost significantly more $^{35}SO_4$-labeled GAG on Days 2 and 4 compared to non-exposed controls. "*" symbol illustrated the significant increase in $^{35}SO_4$-labeled GAG in media for the ITS+IL-1β compared to FBS+IL-1β and ITS at Day 2. At Day 4, ITS+IL-1β had significantly more $^{35}SO_4$-labeled GAG compared to ITS, FBS and FBS+IL-1β. Finally, at Day 8 ITS+IL-1 β had significantly more $^{35}SO_4$-labeled GAG compared to FBS control. Results are reported as mean and bars represent the standard error of the mean. Level of significance used 0.05.

3.1.4 $^{35}SO_4$-labeled GAG in cartilage

At the termination of the study MEDIA had a significant effect (F test, P=0.0173) in $^{35}SO_4$-labeled GAG left in cartilage. ITS+IL-1β samples presented significantly more $^{35}SO_4$-labeled GAG compared to FBS+IL β (P=0.0229. Figure 3.8).

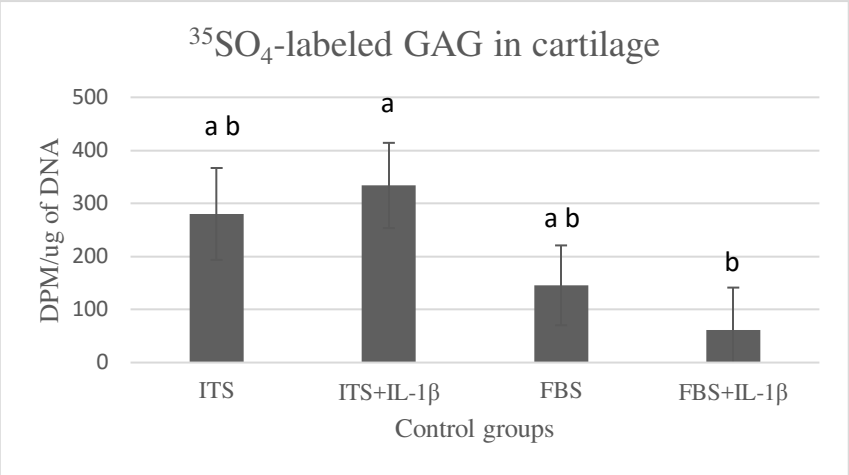

Figure 3.8: $^{35}SO_4$-labeled GAG in cartilage control samples. ITS+IL-1β were significantly higher only compared to FBS+IL-1β controls. Level of significance used 0.05.

3.2 CS treatments compare to control (analysis 2)

This analysis was done to assess the effect of CS in cartilage under inflammatory condition. CS treated samples were compared to ITS controls both under inflammatory condition. The outcome parameters collected were total GAG and $^{35}SO_4$-labeled GAG content in cartilage and media. In this analysis, only the main effect of Treatment was assessed.

3.2.1 Media GAG

Significant effect of Treatment was observed only at Day 2 of the experiment (F test, P=0.0133). ITS+IL-1β control presented increase GAG levels in media compared to Frozen CS (Figure 3.9).

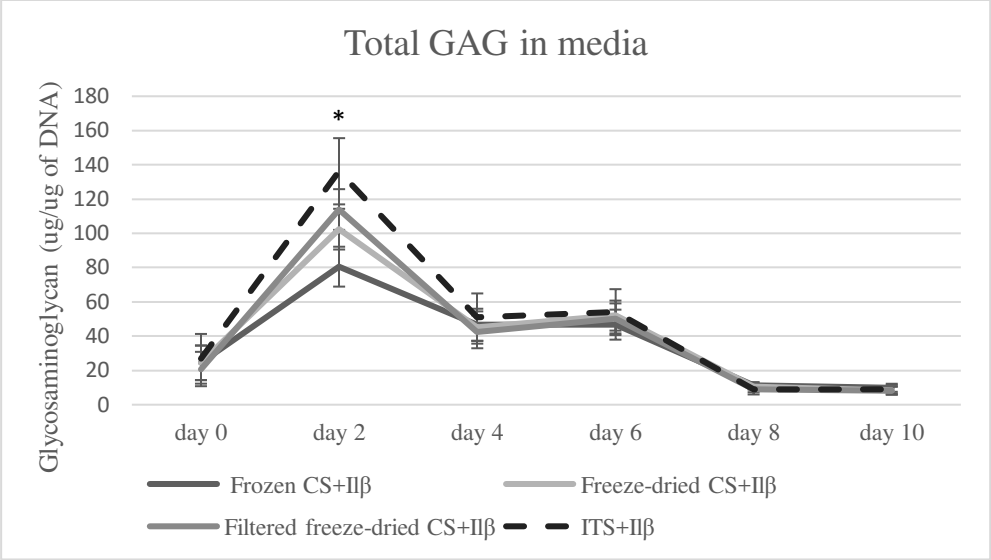

Figure 3.9: Total GAG in media during the experiment for CS treated samples compared to controls. A significant effect of Treatment was observed at Day 2 of the experiment, and ITS+IL-1β had significantly more GAG in media compared to Frozen CS samples, significance illustrated by the "*" symbol. Level of significance used 0.05.

3.2.2 Total GAG in cartilage

No significant differences were observed in total GAG in cartilage.

3.2.3 $^{35}SO_4$-labeled GAG in media

The Treatment effect was significant (F test, P=<0.0001) for $^{35}SO_4$-labeled GAG in media at Day 2, and increase in levels of $^{35}SO_4$-labeled GAG were observed in samples treated with freeze-dried and filtered freeze-dried CS in comparison with ITS+IL-1β control (Figure 3.10).

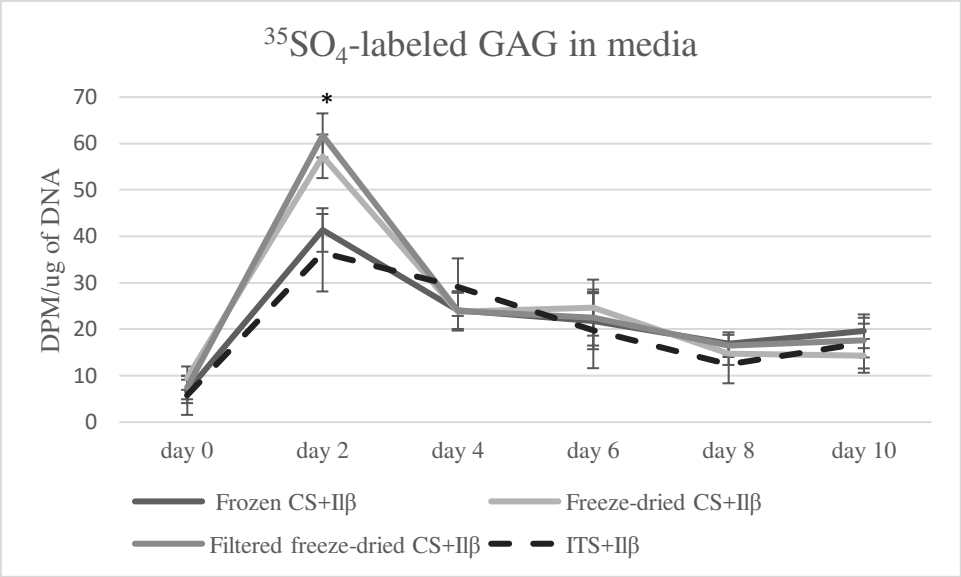

Figure 3.10: $^{35}SO_4$-labeled GAG in media during the experiment samples treated with CS compared to controls. A significant effect of Treatment was observed at Day 2 of the experiment. Filtered freeze-dried and freeze-dried treated samples had significantly more $^{35}SO_4$-labeled GAG in media compared to ITS+IL-β control, illustrated by the "*" symbol. Level of significance used 0.05.

3.2.4 $^{35}SO_4$-labeled GAG in cartilage

The $^{35}SO_4$-labeled GAG left in cartilage at the termination of the study did not present statistical differences between CS treatments and ITS+IL-1β control.

3.3 Study of CS effects (analysis 3)

In this analysis, only CS treated samples were considered to assess the main effects of CS. Outcome parameters evaluated were total GAG and $^{35}SO_4$-labeled GAG in media and cartilage explant as well as gene expression. The main effects evaluated were Treatment, Allogenicity and Concentration.

3.3.1 Media GAG

During the experiment the effect of Treatment was significant at Day 2 (F test P= 0.0519. Figure 3.11). Frozen CS presented lower media GAG levels compared to freeze-dried and filtered freeze-dried CS (P= 0.00175). At Day 4 and 6 of the experiment, Allogenicity had significant effect (F test, P<0.022). Autologous CS treated samples presented more media GAG compared to samples treated with Allogeneic CS (Figure 3.12).

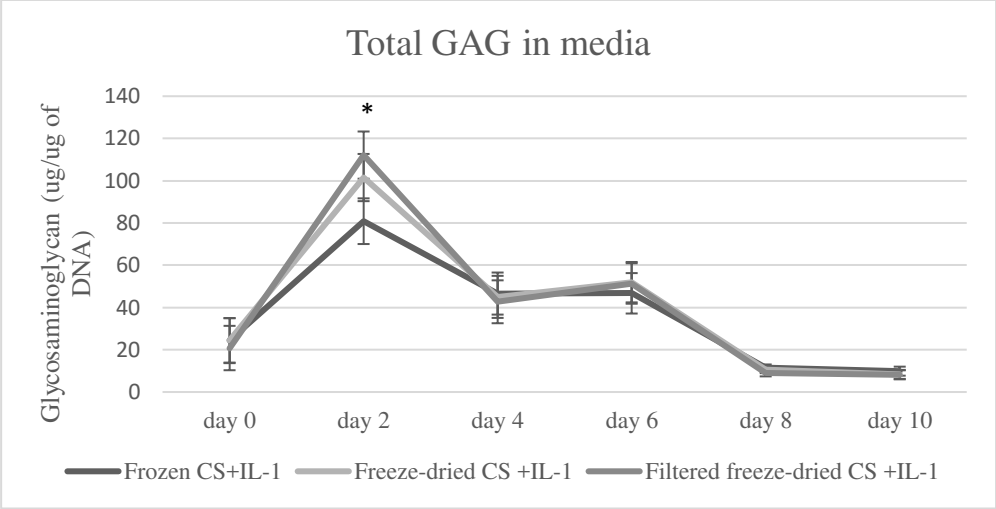

Figure 3.11: Total GAG in media for CS Treatment main effect, averaged over Concentration and Allogenicity. At day 2 of the experiment, the Frozen CS demonstrated significantly lower GAG levels compared to the Filtered freeze-dried and Freeze-dried CS groups, illustrated by "*" symbol. This difference was not observed in the other days of the experiment. Level of significance used 0.05.

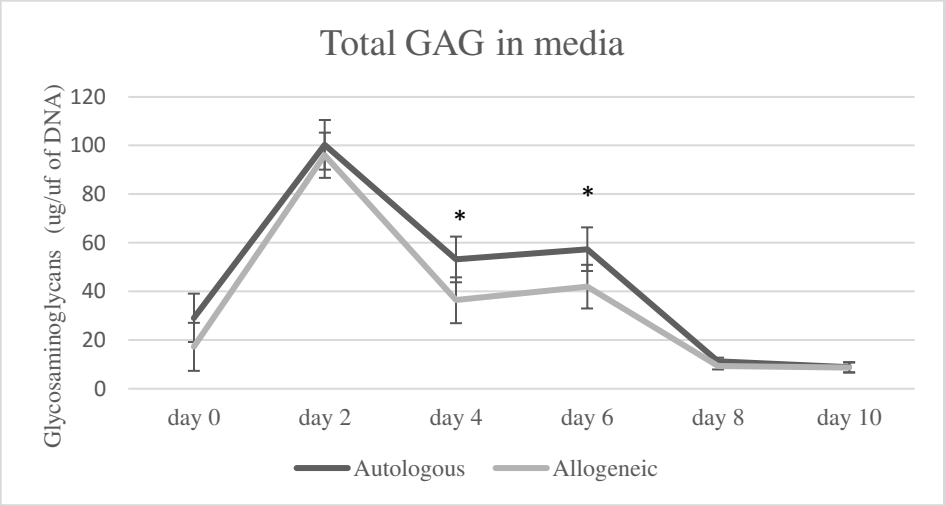

Figure 3.12: Total GAG in media for the Allogenicity main effect averaged over Treatment and Concentration. Samples treated with autologous CS at Days 4 and 6 had significant more GAG in media compared to allogeneic CS treated samples. Level of significance used 0.05.

3.3.2 Total GAG in cartilage

Lack of significance was observed for the comparisons of the main effects for total GAG in cartilage at the end of the experiment.

3.3.3 $^{35}SO_4$-labeled GAG in media

For the evaluation of $^{35}SO_4$-labeled GAG in media, statistical significance was observed for CS Treatment main effect at Day 2 of the experiment (F test, P= 0.0029). Frozen CS treated samples, in both autologous and allogeneic conditions had significantly lower levels of $^{35}SO_4$-labeled GAG in media compared to filtered freeze-dried CS treated samples in both conditions (Figure 3.13). At Day 10, the Allogenicity main effect was significant (F test P= 0.0283). Allogeneic frozen CS had significantly more $^{35}SO_4$ –labeled GAG in media compared to autologous frozen and Freeze-dried CS (Figure 3.14).

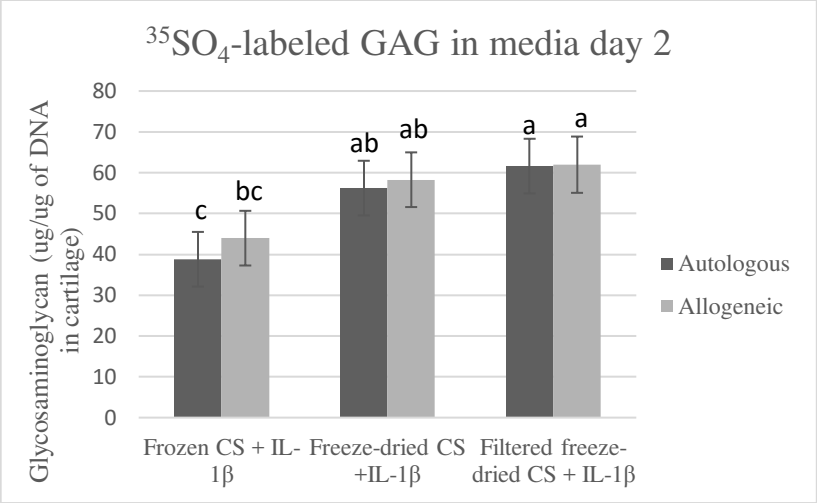

Figure 3.13: $^{35}SO_4$ -labeled GAG in media day 2 of the experiment for the Treatment*Allogenicity interactions. Both autologous and allogeneic filtered freeze-dried CS were significantly increased for $^{35}SO_4$ -labeled GAG in media compared to autologous and allogeneic frozen CS. Level of significance used 0.05.

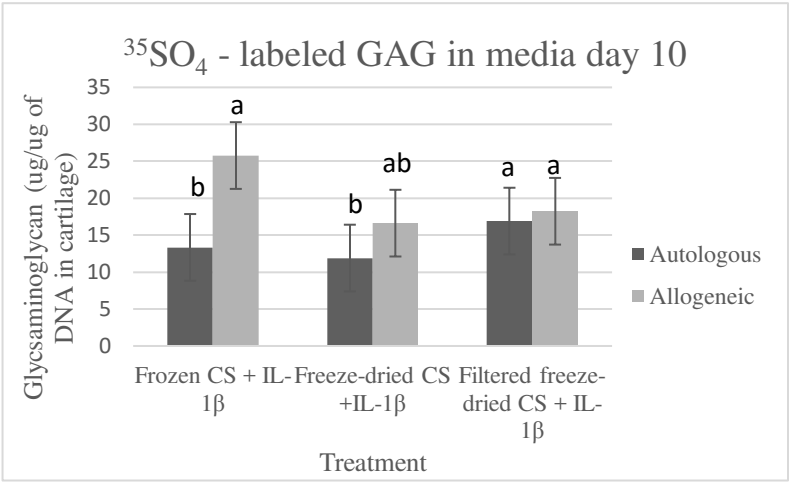

Figure 3.14: $^{35}SO_4$ - labeled GAG in media day 10 for Treatment*Allogenicity interaction. Allogeneic frozen CS treated samples had significantly more $^{35}SO_4$ - labeled GAG in media compared to autologous frozen and freeze-dried CS. Level of significance used 0.05.

3.3.4 $^{35}SO_4$-labeled GAG in cartilage

$^{35}SO_4$- labeled GAG in cartilage at the termination of the study did not showed statistic differences for any main effects or interactions.

3.3.5 Gene Expression

Catabolic cytokines and enzymes related to OA were evaluated for gene expression in this study to assess the modulatory effects of CS in cartilage and synovium. The relative expression for the genes studied did not present a normal distribution, and thus the data was log transformed to fit the assumption of normality and statistical analysis was performed as described in the material and methods section. For ease of presentation and interpretation, the raw fold changes are presented.

3.3.5.1 ADAMTS-5

All cartilage samples treated with different CS demonstrated a mild downregulation of ADAMTS-5 expression although less then 1-fold relative to ITS+IL-1β. Thus, the biologic relevance of gene expression of ADAMTS-4 and ADAMTS-5 in this experiment is suspect.

3.3.5.2 ADAMTS-4

The cartilage explants treated with CS presented a mild downregulation for ADAMTS-4 expression, however less than 1-fold relative to ITS+IL-1β control and consequently the main effects and interactions for the expression of this genes were not biologically relevant.

3.3.5.3 MMP-1

For the gene expression of MMP-1, cartilage samples treated with CS presented substantial upregulation (up to 101-fold) relative to ITS+IL-1β control. Still, none of the main effects or interactions in this experiment had significant effect for this gene.

3.3.5.4 COX-2

Cartilage

Samples treated with different CS demonstrated a slight increase in upregulation for COX-2 expression (less then 2-fold) for all the variables. No significance in any of the experiments main effects or interactions was observed for the expression of COX-2.

Synovium

No significant differences were observed for the comparisons of the main effects for gene expression of COX-2 in synovium explants (Table 3.2).

Table 3.2. Gene expression for COX-2 in synovium. No significant effect of the main effects or interactions was observed in this gene. Different letters denote statistical difference.

Main Effects		Gene Expression of COX-2 in Fold Change (relative to FBS+IL-1β)	SEM
Treatment	**Frozen +IL-1β**	0.61^{a}	0.11
	Freeze-dried +IL-1β	0.81^{a}	0.12
Allogenicity	**Autologous**	0.71^{a}	0.12
	Allogeneic	0.78^{a}	0.12
Concentration	**10%**	0.71^{a}	0.11
	30%	0.71^{a}	0.13

3.3.5.5 IL-1β

Cartilage

Samples treated with CS demonstrated upregulated for the expression of this gene relative to ITS+IL-1β however, less than 1-fold. Comparisons between the different CS Treatment, Concentration or Allogenicity did not demonstrate a significant difference in expression.

Synovium

No statistical significance was observed for the effects of Treatment, Concentration or Allogenicity for the expression of IL-1β (Table 3.3).

Table 3.3. Gene Expression for IL-1β in synovium. No significant effect of the main effects or interactions was observed in this gene. Different letter denote statistical difference.

Main Effects		Gene Expression of IL-1β in Fold Change (relative to FBS+IL-1β)	SEM
Treatment	**Frozen +IL-1β**	4.06[a]	1.84[a]
	Freeze-dried +IL-1β	6.87[a]	2.23[a]
Allogenicity	**Autologous**	6.11[a]	2.17[a]
	Allogeneic	4.14[a]	1.89[a]
Concentration	**10%**	4.45[a]	1.92[a]
	30%	6.38[a]	2.15[a]

4. Discussion

Regarding the evaluation of IL-1β effect in this study (analysis 1), significant increase in total GAG and $^{35}SO_4$-labeled GAG in media was observed after cartilage was exposed to IL-1β, independent of the type of media supplementation used (FBS or ITS). Furthermore, cartilage explants stimulated with IL-1β had significantly less GAG compared to non-stimulated explants. The same trend was observed in FBS supplemented cartilage for the individual comparisons although the main effect of IL-1 β was not significant. Such results are not in accordance with previous findings, where FBS supplemented samples stimulated with IL-1β presented significant matrix catabolism [13, 18]. In the mentioned studies though, cartilage explants were continuously stimulated with IL-1β in oppose to this experiment, where acute stimulation of IL-1β was used (day 0 and 4). It's possible that the acute presence of IL-1β was not enough to induce significant changes in matrix of cartilage samples supplemented with FBS. Thus, although not all outcome parameters presented significant effect of IL-1 β, overall the data in this experiment suggests a

significant catabolic effect of this cytokine. The significant effect of IL-1β in media observed independent of the type of supplementation used support the model in this study.

Increase in total GAG and $^{35}SO_4$-labeled GAG in cartilage was observed in samples supplemented with ITS compared to FBS in this experiment. The concentration of $^{35}SO_4$-labeled GAG left in cartilage at the termination of the study was used in this experiment as an indirect estimation of newly synthetized GAG degradation. After inflammatory insult, catabolic changes in cartilage lead to matrix degradation and GAG loss [19, 20]. Thus, statistically increased $^{35}SO_4$-labeled GAG observed in cartilage supplemented with ITS compared to FBS indicates overall, less degradation for ITS supplemented samples. Such results were unexpected and not in accordance with previous findings [9], once serum-starvation is usually related to cell stress and decrease in cellular basal activity [21]. Still, components present within ITS such as insulin, transferrin and selenium have anabolic properties that could potentially explain these results [10, 22] [23]. Differences between serum and serum-free media were expected in this experiment; however, the detailed investigation for the justification of such differences are beyond the scope of this study. Still, IL-1 β effects was significant independent of the type of media used was in accordance with previous studies [13, 24] and thus was efficient to induce catabolism in this in vitro model.

When comparing the CS treated groups in cartilage to ITS+IL-1β controls (analysis 2), findings suggest that, overall, CS treatments were not effective in protecting cartilage from the catabolic stimulus induced by IL-1β. While little evidence of protective effect of ACS in cartilage was observed in this study, a significant clinical and histological improvement was detected in vivo, in joints with OA [7, 25]. Such results may be justified by the cytokines present in CS used in this study. It has been reported that IL-1Ra:IL-1β ratio needs to range between 10 to

100-fold to inhibit the bioactivity of IL-1β [26]. Therefore, in this experiment the concentration of IL-1Ra in CS should be at least 100 ng/mL to block the stimulus of the IL-1β dosage used (10 ng/mL). Hence, it is possible that the CS produced in this study did not present sufficient levels of anti-inflammatory and anabolic factors to counteract the effects of IL-1 β.

The cytokine profile of CS was not assessed in this study but in previous analysis performed in our lab, the CS produced with the same protocol as used in this experiment demonstrated IL-1Ra concentrations ranging from 11.122 to 50.11 ng/mL (data not shown). These concentrations were similar to what has been reported in commercial kits [8]. This range of IL-1Ra is only up to 5x greater than the concentration of IL-1β used and thus, would not be able to block the effects of this cytokine efficiently. Additionally, it's important to consider that 10 ng/mL of IL-1 β is a supra-physiological concentration beyond of what was found in joints with arthritis (ranging from 25 pg/mL to 175 pg/mL) [27]. Therefore, the lack of protective efficiency in cartilage matrix observed particularly with the use of Frozen CS does not imply inefficiency of the treatment in vivo or in other culture systems.

When considering the main effect of Treatment (analysis 3) specifically at day 2 of the experiment, increased levels of total and $^{35}SO_4$-labeled GAG in media were observed in freeze-dried and filtered freeze-dried CS compared to control and frozen preparations. In this study, frozen CS was used to represent the current state of practice to compare the effects of the freeze-dried versions of CS. Although, significant increase in total GAG and $^{35}SO_4$-labeled GAG in media after inflammatory insult compared to frozen preparations, differences between CS treatments were not noticed in any other day of the experiment in media or cartilage explants. Hence, even if lyophilization interfered with the cytokine profile of CS, such differences did not play a significant role in cartilage after 10 days of culture in the model used.

The main effect of Concentration was accessed in this experiment (analysis 3). Two concentrations of CS were studied in this experiment, 10% and 30% in media. No significance was found for GAG or $^{35}SO_4$-labeled GAG in cartilage or media during the experiment, which suggests that the effects of CS in cartilage were equivalent for the two concentrations used in this experiment. These findings are in accordance with previous research in which the total GAG present in chondrocyte pellets stimulated with IL-1β did not demonstrate to be significant with the use of different ACS concentrations [28].

Significant effect of Allogenicity was observed in media (analysis 3) at Days 4, 6 and 10 of the study. Such effect was variable depending upon the assay and day of the experiment. In addition, no significant effect of Allogenicity was observed in cartilage. Hence, for most of the comparisons, no significant difference was observed suggesting similar effects of Autologous and Allogeneic CS in this study. Up to date no studies comparing the effects of autologous and allogeneic conditioned serum have been published. Regarding to other biological therapies though, the use of allogeneic presentations of PRP demonstrated to promote similar bone regeneration compared to autologous forms, in a rabbit model [29]. In the current study, for most of the comparisons evaluating autologous and allogeneic effects of CS in cartilage and media no significant changes were observed suggesting equivalent effects of both conditions, which was also noted in previous studies using allogeneic blood-derived products[29].

Several cytokines and proteolytic enzymes are known to have its synthesis and activation induced by IL-1β stimulation in chondrocytes [20, 30]. Thus, to access the potential modulatory effects of CS in downstream cytokines induced by IL-1 β, gene expression of ADAMTS-5, ADAMTS-4, COX-2, MMP-1 and IL-1β were studied in cartilage.

ADAMTS-4 and ADAMTS-5 are generally associated with catabolic changes in cartilage being frequently investigated in in vitro studies [31]. In this experiment, CS did not significantly interfere with gene expression of ADAMTS-5 and ADAMTS-4 in cartilage explants. MMP-1 on the other hand, was strongly up-regulated (up to 101-fold) in samples treated with CS (averaged over Concentration and Allogenicity) compared to ITS+IL-1β controls in cartilage. MMP-1 demonstrated to be more upregulated in cartilage stimulated with IL-1β in vitro then cartilage under early degeneration in vivo [32] [30]. Thus, the significant up-regulation of MMP-1 in this experiment might over estimate what would be observed with the use of CS in vivo.

In joints with OA, the intra-articular increase of inflammatory mediators such as IL-1β and COX-2 induce the increased in pro-inflammatory cytokines and enzymes leading to oxidative stress [33]. IL-1β relative expression was mildly downregulated in cartilage, however such low expression of this gene was not biologically relevant. Indeed, previous studies have demonstrated low expression levels of IL-1β, even only 24 hours after mechanical injury [34]. In addition, COX-2 relative expression in cartilage was not biologically significant. These findings suggest that CS did not have a significant effect in expression of COX-2 and IL-1β in cartilage at the termination of the study.

In synovium, the gene expression of IL-1β and COX-2 was not significant for the main effects of Treatment, Allogenicity and Concentration. Although synovium treated samples had ITS as media supplementation, further evaluation of the controls utilized in this experiment (data not shown) suggested that ITS+IL-1β controls possibly had significant reduced cellular viability compared to CS treated samples and FBS controls. For this reason, FBS+IL-1β was used as a calibrator for the gene expression data of synovium samples treated with CS. No significant effect of Treatment, Allogenicity or Concentration for gene expression of COX-2 or IL-1β was

observed. Such findings suggest that the different CS used in this study had equivalent effect in gene expression in synovium.

The up-regulation of gene expression in cartilage for MMP-1 observed in this experiment, could suggest a potential pro-inflammatory effect of CS treatment in cartilage; however, such findings were not observed in the gene expression of other catabolic enzymes evaluated in this study or in the biochemical analysis of cartilage explants and media. Consequently, overall the CS treatments did not have a significant effect in gene expression in cartilage and did not present a protective effect against IL-1β in this experiment.

It is possible that frozen CS might have a modulatory effect in cartilage under inflammatory conditions however, not efficient enough to inhibit the degradative effects of IL-1β in this study as discussed. The biochemical data in media obtained after stimulation with IL-1β at day 2 suggests that lyophilization might have interfered with CS components. However, such differences were not observed in any other days of the study or cartilage samples, suggesting overall similar effects of the difference CS treatments.

5. Conclusion

Although the CS modulatory effects were not efficient in reducing the catabolic effects of IL-1β in the model used, these results do not imply inefficiency of this product in other models or in vivo, once the dose of IL-1β used here was greater than observed in joints. Overall the use of different CS treatments, in autologous or allogeneic conditions and in different concentrations demonstrated to be equivalent and only the hypothesis 3 of the study was rejected, once CS effects were not concentration dependent in this study. Finally, the different formulations of CS used had similar modulatory effect in cartilage and synovium.

The allogeneic freeze-dried version of CS proposed in this study demonstrated to be equivalent to the current state of practice used (frozen ACS) and could offer a more homogeneous and user-friendly option for use of biological therapies. Further investigation in in vivo models to evaluate the modulatory effects of allogeneic freeze-dried CS in joints are warranted to confirm these results.

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SUMMARY AND CONCLUSIONS

This project had as main objective to compare the effects of Allogeneic Freeze-dried PL and CS to the current state of therapy (autologous fresh/frozen preparations) in cartilage and synovium under inflammatory condition in vitro. An off-the-shelf allogeneic version of such therapies would enable the immediate availability of a more homogeneous, stable and user-friendly product for clinical use.

The effects of different formulations of PL and CS were assessed in an in vitro model of osteoarthritis. PL and CS were evaluated in different treatments (frozen, freeze-dried and filtered freeze-dried), concentrations (PRP: 1.5x, 3x and 6x/ CS: 10% and 30%) and in autologous and allogeneic conditions (Allogenicity). To evaluate the protective and modulatory effects of PL and CS, biochemical and gene expression analysis were done in cartilage. Total GAG and $^{35}SO_4$-labeled GAG in cartilage and media were evaluated as a measure of synthesis/ degradation (of newly synthetized GAG) in cartilage. Gene expression of pro-inflammatory enzymes and cytokines in cartilage and synovium (CS experiment) were accessed to evaluate modulatory effects of PL and CS therapies.

Freeze-dried and frozen preparations of CS and PL failed in protecting cartilage and synovium from inflammatory stimulus in the model used. In fact, PL presented upregulation of pro-inflammatory enzymes and/or cytokines in cartilage, suggesting potential pro-inflammatory effect. However, the gene expression obtained in cartilage at day 10 of the study for PL, did not reflect the changes observed in cartilage matrix. These findings suggest that PL's multiple cytokines might have influenced other mechanisms not at the gene expression level or not

evaluated in this experiment that could have influenced in cartilage matrix homeostasis, explaining the discrepancies observed between cartilage matrix data and gene expression.

The lack of protective efficiency observed for biological therapies implemented in this study could be explained by several factors. For PL, the use of freeze-thawing to activate the platelets could have played a role in this product's efficiency in that the partial lyse of platelets could have led to a lower growth factor content that could not overcome the negative effects caused by IL-1β stimulation. It is possible that the addition of a biochemical stabilizer for platelets and the implementation of other activation method could have led to different results. Freeze-drying PL demonstrated an effect in cartilage gene expression. However, although both Frozen and freeze-dried preparations seemed to regulate gene expression differently, it did not reflect in significant changes in matrix at the end of the experiment. Freeze-drying CS seemed to potentially interfere with its properties increasing the catabolism observed at Day 2. However, such effects were not observed in any other days of the experiment or in cartilage or synovium explants at the termination of the study, suggesting overall similar effects of different CS treatments.

Product concentration had a significant effect in this study. PL dose-dependent efficiency curve was not linear, and cartilage treated with 3x concentration had more $^{35}SO_4$-labeled GAG compared to cartilage treated with PL in 1.5x and 6x concentration. Such findings suggest less catabolism for samples treated with PL in 3x concentration. In CS, the increase in concentration did not demonstrate a significant effect in cartilage or synovium.

Finally, regarding to the allogenicity, allogeneic and autologous conditions demonstrated to be equivalent for CS but not PL. Autologous PL demonstrated increased gene expression of

IL-1β compared to allogeneic PL. Such differences were not observed for the other genes or in the biochemical analysis.

Therefore, we reject the three hypotheses of this study. The first hypothesis was rejected because freeze-dried PL did not demonstrate equivalent effect compared to frozen preparations. Freeze-dried PL did modulate gene expression differently compared to frozen, although the effect in cartilage matrix was equivalent. For CS, freeze-drying may have affected CS cytokines; however, it did not present a significant effect during the experiment or in cartilage or synovium at the termination of the study. The second hypothesis was rejected because allogeneic PL was not equivalent to autologous in gene expression analysis. Overall with CS, both autologous and allogeneic conditions were demonstrated to be equivalent. Lastly, this study demonstrated a concentration-dependent effect of PL, but this was not seen with CS.

In conclusion, allogeneic freeze-dried PL demonstrated to modulate inflammation differently compared to the current state of practice used, however such effects did not interfere with cartilage matrix metabolism. Further investigation of the effects of allogeneic freeze-dried PL in synovium in vitro, and in vivo OA models are warranted to confirm these results. With CS, the proposed allogeneic freeze-dried version of the product demonstrated to be equivalent to the current state of practice, offering a more homogeneous and user-friendly product for use. The investigation of allogeneic freeze-dried CS in in vivo models to evaluate the modulatory effects of this product in joints is necessary to confirm these results.

APPENDIX

The Paw Classification System for the PL used in this study

The PAW classification system uses 3 components for its classification; absolute number of platelets, method for platelet activation and presence or absence of white blood cells. Briefly, platelets counts are classified as follow: P1= bellow or equal to baseline; P2= number of platelets above baseline until 750,000/ μL; P3= between 750,000 and 1,250,000/ Ml[1]and finally P4= above 1,250,000 platelets/ μL. For the activation method, the PRP is classified as endogenously activated (which does not need to be indicated in the classification) and exogenously activated (indicated with an "X"). In this experiment, PRP samples were frozen and thaw before application for storage and activation of the platelets, and thus could be considered as exogenously activated. The WBC counts can be classified as: A=above baseline and B= bellow baseline[1]. The classification of the different dilutions of the 1 x PRP used in this study can be classified as follow on Table 1.

Table 1: "PAW" classification system for the PL obtained in the study. The PAW system classifies PRP according to 3 components; absolute number of platelets, method for platelet activation and presence or absence of white blood cells.

Platelet Product used in this study	"PAW" classification system
1.5 x PL	P2-X-B
3 x PL	P3-X-B
6 x PL	P4-X-B

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