ABSTRACT

JENIO, FRANK ZACKARY. The Past, Present and Future of Equine Orthobiologics: Platelet-derived Peptide(s) to treat Osteoarthritis. (Under the direction of Dr. Lauren V. Schnabel).

Osteoarthritis (OA) is a common yet debilitating degenerative joint disease contributing to lameness in the majority of equine populations. OA is perpetuated in the joint from synoviocytes and chondrocytes inability to maintain homeostasis because of an increase in catabolic products like matrix metalloproteinases and interleukin 1β and a decrease in anabolic products like collagen and hyaluronic acid. The field of regenerative medicine has sought out a solution that goes beyond masking symptoms, but influences protein production in order to reverse the inflammatory feedback loop between the two cell types. Preliminary data from the research team shows BIO-PLY, a pooled and fractionated platelet-rich plasma lysate, is a superior option to steroids like triamcinolone acetonide, hyaluronic acid supplementation and traditional PRP *in vitro.* A major goal in the translation of BIO-PLY to clinical use is the potential to identify the bioactive peptides within that can be synthesized to mimic the effects without the required production of a biologic. The next step in BIO-PLY research is a clinical trial; however, due to its allogeneic design as a pooled product, approval for clinical trials is a more rigorous challenge. The BIO-PLY-derived peptides, as determined by mass spectrometry and de novo sequencing, may serve as a synthetic alternative to create off-the-shelf disease-modifying therapeutics. Four of these peptide classes were selected, CXCL4, CXCL7, CCL5 and Tβ4, and were deemed as lead peptides based on their human availability and large frequency of equine specific peptides that fall under their motif. These serve as the primary experimental treatments to be analyzed by the research team.

The purpose of this thesis is to (1) contextualize OA in equine models, describe the timeline of important biological therapeutics and their related studies that have been completed to lead to the creation of BIO-PLY, (2) review the broad literature of biological therapeutic clinical trials to discern the common shortcomings and limitations to be avoided in addition to using those themes as guidance to devise a clinical trial for a platelet-derived product in equine populations, and (3) explore the efficacy of four BIO-PLY-derived lead peptides both in combination and in isolation as a treatment for LPS-induced OA monocultures of synoviocytes and chondrocytes.

The research team have designed the general framework of a prospective, double-blind, matched pairing, controlled randomized multicenter equine clinical trial complete with power analysis, inclusion and exclusion criteria, and grading and scoring systems. Moreover, future research teams can use this document as a translational blueprint when designing orthobiologic clinical trials with human, equine or other subjects in order to avoid the common pitfalls and yield the best data possible. Preliminary findings from the BIO-PLY-derived lead peptides show that treatment with these lead peptides of LPS-stimulated synoviocytes preserved cell count and viability, maintained *HAS2* gene expression and downregulated *IL-1β* expression to that of the steroid control. For LPS-stimulated chondrocytes, lead peptides downregulated *IL6* gene expression and maintained *COL2* gene expression. The results of this study support continued investigation into platelet-derived peptides for the treatment of osteoarthritis.

The Past, Present and Future of Equine Orthobiologics: Platelet-derived Peptide(s) to treat Osteoarthritis

by
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DEDICATION

To science

BIOGRAPHY

F. Zack Jenio was born in Tacoma, Washington, USA and moved around the country as a military child before uprooting and living in Dubai, United Arab Emirates. During high school, he was involved with research outside of the classroom with Dr. William Murrell where he assisted in the writing of the chapter “Complete Rotator Cuff Tear: An Evidence-Based Conservative Management Approach” in the book “Advances in Shoulder Surgery”. This sparked Zack’s interest in regenerative medicine and novel biologic treatments such as platelet-rich plasma.

Upon graduation from high school in 2018, Zack returned to the US to attend North Carolina State University as a Park Scholar. He completed his undergraduate degree in Biological Sciences: Integrative Physiology and Neurobiology with a minor in Middle East Studies in spring 2021 as part of an accelerated bachelor’s/master’s program. He then began his master’s in Comparative Biomedical Sciences from the College of Veterinary Medicine and will graduate officially in the summer of 2022. Throughout his time at NC State, Zack has been involved in biomedical research through the Schnabel Equine Sports Medicine Laboratory since his first semester and is completing his MS thesis under the guidance of Dr. Lauren V. Schnabel. Continuing from his previous experiences, Zack was interested in exploring novel orthobiologic treatments for musculoskeletal disorders and worked closely under the mentorship of Dr. Jessica Gilbertie where he began his culminating project on BIO-PLY-derived peptides. Outside of the Schnabel Lab, Zack also completed research through the Jacob Lab regarding clinical surgical site infections and Arabic sociolinguistics research through the department of foreign languages

and literatures. After the completion of his master's program, Zack intends to pursue a career in public health with an emphasis on community-based interventions and general wellbeing.

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Without all of these wonderful people, and so many more, I would not be the person I am today. I am grateful for every interaction I was able to have and attribute a large part of my success to

all of the support I received. As I leave Raleigh, I know I take a little piece of everyone with me as I head out into the world and start my life.

TABLE OF CONTENTS

LIST OF TABLES ix

LIST OF FIGURES x

Chapter 1: Contextualizing Osteoarthritis and the History of BIO-PLY 1

Abstract 1

1.1 | The Equine Model for Osteoarthritis 2

1.2 | Platelet-rich Plasma and BIO-PLY 4

1.3 | Conclusion 12

Chapter 2: Chapter 2: Drafting the Next Steps for Orthobiologic Research: A Future Clinical Trial 14

Abstract 14

2.1 | Rationale 15

2.2 | Thematic Review of Select Clinical Studies 16

2.3 | Study Design: Overview 20

2.4 | Study Design: Specific Parameters 22

2.5 | Conclusions 27

Chapter 3: Preliminary viability and Nanostring nCounter™ analysis of BIO-PLY-derived lead peptides in cell monocultures show promise, prompt further discussion and investigation 30

Abstract 30

3.1 | Background 32

3.2 | Methods 36

3.3 | Results 40

3.4 | Discussion 47

3.5 | Funding 52

3.6 | Acknowledgements 52

Chapter 4: Conclusions and Next Steps 56

References 58

LIST OF TABLES

Table S2.1 Review of human and equine clinical trials 76

Table S2.2 Lameness grading rubric 81

Table S2.3 Radiographic scoring rubric 82

Table S2.4 Fetlock joint effusion grading rubric 83

Table S2.5 Mathematical symbols definitions 84

Table S3.1 Nanostring nCounter™ Codeset Probes Design 85

LIST OF FIGURES

Figure 1.1 BIO-PLY significantly upregulates HAS1 and HAS2 gene expression in synoviocytes 8

Figure 1.2 BIO-PLY significantly increases hyaluronic acid (HA) production in co-culture 9

Figure 1.3 BIO-PLY significantly decreases IL-1β and MMP-13 gene expression in cultured synoviocytes. 10

Figure 1.4 BIO-PLY significantly increases COL1A1 and COL2A1 gene expression without altering the COL2A2:COL1A1 ratio 11

Figure 3.1 Schematic of study design 36

Figure 3.2 Lead peptides, in combination, preserve synoviocyte cell count and viability, but not for chondrocytes. 43

Figure 3.3 CXCL4 and CXCL7, in isolation, maintain synoviocyte HAS2 gene expression compared to steroid but no treatments alter HAS1 or HAS3 gene expression 45

Figure 3.4 Treatment with all four lead peptides significantly downregulates synoviocyte IL-1β gene expression, but neither MMP-3 nor -13 gene expression 47

Figure 3.5 Treatment with all four lead peptides decreases chondrocyte IL6 gene expression, but not IL-1β gene expression 48

Figure 3.6 CXCL4 and CXCL7, in isolation, maintain chondrocyte COL2 gene expression compared to the steroid, but not COL1 5-

CHAPTER 1

Contextualizing Osteoarthritis and the History of BIO-PLY

ABSTRACT

Osteoarthritis (OA) is a common yet debilitating degenerative joint disease contributing to lameness in the majority of equine populations. OA is perpetuated in the joint from synoviocytes and chondrocytes inability to maintain homeostasis because of an increase in catabolic products like matrix metalloproteinases (MMPs) and interleukin 1β (IL-1β) and a decrease in anabolic products like collagen and hyaluronic acid. The field of regenerative medicine has sought out a solution that goes beyond masking symptoms, but influences protein production in order to reverse the inflammatory feedback loop between the two cell types. Platelet-rich plasma (PRP) has been heavily researched as a therapeutic for osteoarthritis in both human and equine populations with evidence *in vitro* showcasing significant reduction in catabolic products and increase in anabolic products, while *in vivo* studies have trended towards general short-term benefits of treatment with no definitive evidence due to variable study designs. Limitations of PRP include its difficulty in production, varied composition from donors and conflicting definitions by author and institution; therefore, the need for an improved orthobiologic. Preliminary data from the research team shows BIO-PLY, a pooled and fractionated platelet-rich plasma lysate, as a superior option to steroids like triamcinolone acetonide, hyaluronic acid and traditional PRP *in vitro*. The bioactive peptides within BIO-PLY have the potential to be synthesized to mimic the effects of BIO-PLY without the required laborious production. We speculate that the bioactive peptides with BIO-PLYcould achieve similar effects to their patent pending BIO-PLY. This comprehensive review of the literature contextualizes the pathophysiology of OA, the timeline of novel therapeutics to treat the disease and major studies crucial to the development of the BIO-PLY-derived treatment.

1.1 | The Equine Model for Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease (DJD) that results from the loss of articular cartilage and specifically is the summation of degenerative disorders between the cells and extracellular matrix of the synovium, the connective tissue lining the joint capsule, that alter the composition of bone, ligaments, nerves and periarticular muscle (1–4). These structural changes within the joint space take the form of bone remodeling, synovial inflammation and cartilage degradation. The degeneration of articular cartilage with fibrillation, fissure and ulceration thereby expose the bone underneath and continued injury further leads to complications including chondrocyte cell death, increased degradation of the cartilage matrix and cartilage, and complete exposure of the underlying bone (3–6).

More than 80% of people over the age of 55 exhibit some evidence of degenerative joint disease with 10-30% experiencing significant pain and impact in daily activities (5). Not only are DJDs present and a significant health concern in human medicine, but also in equine health as OA accounts for 60% of lameness in adult horses (3,7). Moreover, horses have a naturally occurring progression of osteoarthritis, similar to humans, that has been established to serve as a model for clinical osteoarthritis (8). This allows a humane and relevant model to study the pathogenesis of osteoarthritis from a cellular and molecular perspective in equine patients (9).

OA stems from the inability of chondrocytes to balance anabolic and catabolic activities in regards to the homeostatic balance of maintaining the extracellular matrix (10). Synovitis, or the inflammation of the synovium, contributes to the progression of osteoarthritis as the synovial-like fibroblasts, local macrophages and chondrocytes produce inflammatory cytokines such as, but not

limited to, interleukin-1 beta (IL-1β) and tissue necrosis factor α (TNF-α) which stimulate matrix degrading enzymes (3,6,11). These cytokines work through a positive feedback mechanism to further stimulate the transcription factor nuclear factor kappa B (NF-κB), the nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) inflammasome and caspase-1 to upregulate the production of IL-1β that ultimately contribute to chondrocyte apoptosis (12). Further, these stimulated chondrocytes will release metalloproteinases (MMPs) and aggrecanases (ADAMTS-4) amongst other catabolic enzymes to decrease type II collagen and aggrecan in the extracellular matrix, thereby causing cartilage loss (12,13). MMPs, specifically MMP-3 ,-9 and -13, will cleave proteoglycans and type II and XI collagens to release glycosaminoglycan (GAG) and collagen fragments (14). Inflammation is disseminated within the joint as these cytokines induce their own production and others such as IL-6, IL-8, nitric oxide-NO and prostaglandin (4).

The cytokine-receptor associated molecular patterns are the primary driving force for the cascade of genetic and enzymatic consequences that ultimately lead to the degradation of cartilage matrix in osteoarthritis (15). These inflammatory signals include previously discussed cytokines including IL-1β and TNF-α and their recognition by toll-like receptors (16), complement factors (17,18), other pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (19). As a result of their recognition, the receptors activate NF-κB and other mitogen-activated protein kinases (MAPKs) that encode enzymes, cytokines or other proteins that contribute to cartilage degradation (20). Using the model that recognizes the joint as an organ, there are interactions between the cells that make up the tissue (synoviocytes and chondrocytes) and immune cells, primarily macrophages. The inflammatory cytokines produced by both the organ cells and the immune cells stimulate macrophage linked MMPs to influence the disease

progression in a positive feedback system (15,21,22).

The majority of current treatments for osteoarthritis focus on the treatment of symptoms, thereby increasing interest for biological therapies that are disease-modifying. Due to its anabolic, anti-catabolic and anti-inflammatory effects, platelet-rich plasma (PRP) is an innovative treatment alternative for OA and has been heavily researched over the past decade (4).

1.2 | Platelet-rich Plasma and BIO-PLY

Platelet-rich plasma (PRP) is the concentration of growth factors and peptides from platelets and therapeutic use has grown within veterinary medicine in the field of sports medicine as a treatment for musculoskeletal injuries and disorders (23–28). Intra-articular injections of autologous PRP are hypothesized to have anabolic, anti-catabolic, anti-inflammatory and antimicrobial properties based on the numerous growth factors within platelets and plasma (29–31)**.** Of particular interest is the impact of PRP on synoviocytes and chondrocytes, the two cell types within the joint and the focus of this review.

Research on fibroblast-like synoviocytes (FLS) lack in depth and quality compared to their chondrocyte counterpart (26) although the FLS constitutes about 80% of the normal synovium and heavily interacts with the cartilage through the production of cytokines and matrix metalloproteinases that mediate chondrocyte catabolism. Some studies observed that synoviocytes treated with PRP resulted in a higher prostaglandin E 2 (PGE2) concentration, decreased oxidative stress, increase in *HAS2* gene expression, increase in hyaluronic acid (HA) synthesis and reduction of MMP-13 (32,33). In human models, the treatment of synovial cells with leukocyte rich-PRP

(LR-PRP) resulted in an increase in proinflammatory cytokines and significant cell death (34). For damaged articular cartilage, PRP has been supported as a therapeutic agent and findings show decreased cartilage catabolism, chondrogenic differentiation of pluripotent mesenchymal cells (MSCs) and increased matrix production through endogenous HA gene expression (27,33,35–41). From a biochemical perspective, PRP is hypothesized to have these beneficial effects as the releasate decreases NF-κB activation to downregulate genes for matrix degradation (42)

PRP has shown positive outcomes in patients suffering from OA (43–50) in addition to equine populations (51). Despite numerous studies observing PRP in clinical experiments, controversial results increase the reluctance of its use as an OA treatment (32,52) with positive results observed from equine studies with a higher chance of bias, especially when compared to human studies (53). The major reason for the variability in PRP results is due to the lack of standardized preparation protocol.

There are numerous definitions and classifications for PRP and its related products, but debate continues because of the diverse cellular and cytokine profile with each method of preparation (54,55). Although there is a wide use of PRP within veterinary medicine, no valid standardized classification has been proposed and accepted. The limitations of PRP and its clinical trials will be further explored within this section in addition to *Chapter 3*.

The composition of PRP (platelet concentration, growth factor, cytokine and leukocyte concentrations) differs greatly based on patient factors including age, sex, anti-inflammatory drug (NSAID) use, and for equine populations age, breed, time of day, hydration level, and exercise

have also been found to have effects on PRP generation (41,56–58). Understanding the theory and benefits behind PRP and the various platelet growth factors and bioactive proteins in combination with the limitations of blood donors and patients, there is growing interest in the use of a pooled platelet-rich plasma lysate (PRP-L) for bone and muscle regeneration (18,59–75). PRP-L is an important development because the pooled lysate can be frozen and used allogenicallyin clinical treatment and allows for platelets from donors with quality bioactive components to be used in patients without such components. Some researchers have started testing PRP-L in human clinical trials (74).

A study of particular interest and within the specific scope of this thesis is Gilbertie et al., 2018; because the aim was to examine the cellular effects of a pooled allogeneic platelet-rich plasma lysate (PRP-L) on equine synoviocytes and chondrocytes *in vitro* that have been stimulated to mimic an osteoarthritic joint environment (27). To allow for increased reproducibility, the PRP was pooled and the antimicrobial, anti-inflammatory fraction isolated, which was derived from an acellular PRP lysate enriched for cationic, <10 kDa peptides (termed BIO-PLY, standing for BIOactive fraction of Platelet-rich plasma LYsate). Methods, similar to those in *Chapter 2*, sought to analyze the expression of important anabolic and catabolic genes for both cell types via qPCR in addition to quantifying the concentration of important cytokines, metalloproteinases and hyaluronans within the media. In synoviocytes, the PRP-L increased cellular proliferation, HA production via *HAS2* gene upregulation and anti-inflammatory IL-6 production for both naive and inflamed groups. In chondrocytes, conditioned media from the PRP-L treated synoviocytes decreased gene expression of catabolic matrix metalloproteinases like *MMP-3* and *MMP-13* while increasing the gene expression of anabolic factors such as *COL1*, *COL2*, aggrecan and lubrican.

All findings were consistent with previous studies (27,33,42,76,77). This study concluded that treatment of inflamed synoviocytes and chondrocytes with PRP-L *in-vitro* resulted in the upregulation and production of important anabolic cascades (HA, collagen, aggrecan, IL-6) and downregulation of catabolic factors such as MMP-13. The research team consisting of Drs. Jessica M. Gilbertie, Lauren V. Schnabel and Thomas P. Schaer developed a PRP-L preparation that is nearly 50 times more concentrated with growth factors and bioactive peptides compared to whole blood and the standard PRP which is 2-10 times the concentration of blood (78). This product, patent-pending, as BIO-PLY, has been shown to exhibit anti-biofilm, anti-infection, anti-inflammatory and chondroprotective properties (41,79) making it an excellent option to experiment with for an osteoarthritis biologic.

BIO-PLY is a derivative of PRP-L, and both have been studied *in vitro* by the research team in a model of osteoarthritis (79). Data in-submission from the researcher's lab shows using a co-culture methodology (synoviocytes and chondrocytes) that BIO-PLY is chondroprotective as it significantly increases *HAS* gene expression and hyaluronic acid production expression compared to hyaluronic acid treatment (Hyvisc®) and triamcinolone acetonide corticosteroid (KENALOG®), both common OA therapies (Figures 1.1 and 1.2). The biologic also exhibits anti-inflammatory effects from the significant decrease of *IL-1β* and *MMP-13* gene expression in cultured synoviocytes (Figure 1.3). Finally, BIO-PLY was shown to significantly upregulate *COL1A1* and *COL2A1* expression levels compared to controls and other treatments without the alteration of the COL2A2:COL1A1 ratio (Figure 1.4). The significance of these findings are the simultaneous decrease in pro-inflammatory mediators without compromising the concentrations of anabolic molecules such as HA and collagen. In fact, BIO-PLY upregulates these anabolic genes

significantly whereas a nonspecific steroid inhibitor will downregulate all genes when applied, including beneficial ones. As a result, BIO-PLY is hypothesized to be a superior therapy compared to corticosteroids in addition to being more concentrated and effective than PRP-L.

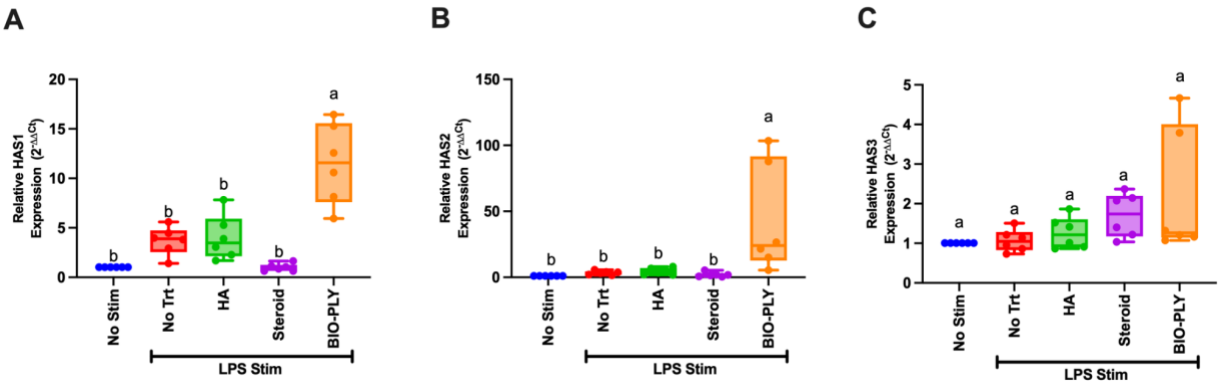


Figure 1.1. BIO-PLY significantly upregulates *HAS1* and *HAS2* gene expression in synoviocytes. Synoviocyte gene expression levels of *HAS1* (A), *HAS2* (B) and *HAS3* (C). Treatment with BIO-PLY resulted in significantly increased synoviocyte HAS1 and HAS2 expression compared to other treatments and controls with no change in HAS3 expression. (No Stim = Non-stimulated control; LPS Stim = LPS stimulation; No Trt = Non-treated control; HA = hyaluronic acid treatment (Hyvisc®); and Steroid = triamcinolone acetonide corticosteroid (KENALOG®). Bars are means and standard deviations of co-cultures from 6 horses and significant differences ($p<0.05$) between treatments are indicated by differing letters as determined by ANCOVA with Tukey post-hoc.)

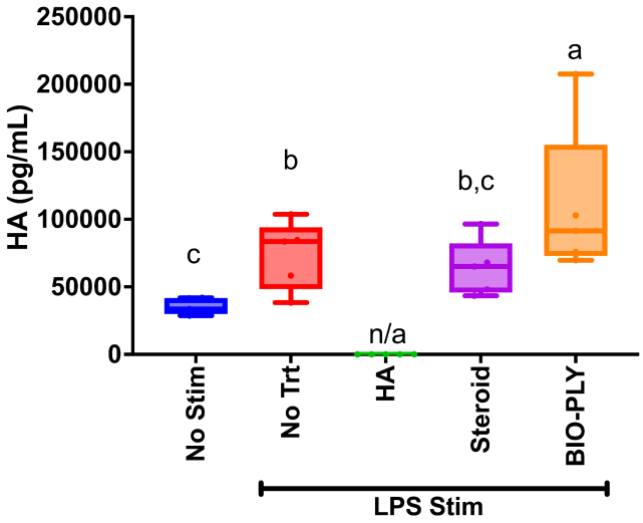


Figure 1.2. BIO-PLY significantly increases hyaluronic acid (HA) production in co-culture. Production of HA measured in the co-culture media 72hr post treatment using a commercial ELISA kit. Treatment with BIO-PLY resulted in significantly increased HA concentrations in the media compared to all other groups assessed. (No Stim = Non-stimulated control; LPS Stim = LPS stimulation; No Trt = Non-treated control; HA = hyaluronic acid treatment (Hyvisc®); and Steroid = triamcinolone acetonide corticosteroid (KENALOG®). Bars are means and standard deviations of co-cultures from 6 horses and significant differences ($p<0.05$) between treatments are indicated by differing letters as determined by ANCOVA with Tukey post-hoc.)

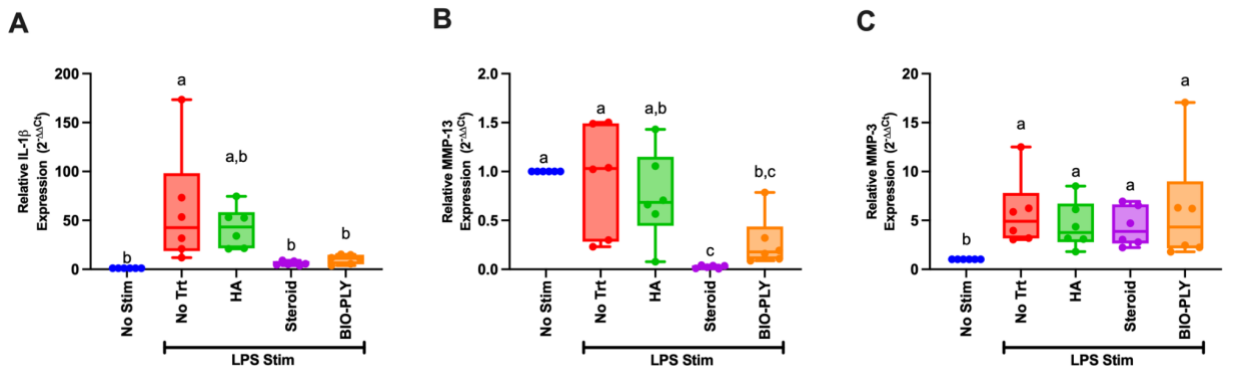


Figure 1.3. BIO-PLY significantly decreases *IL-1β* and *MMP-13* gene expression in cultured synoviocytes. Synoviocyte gene expression levels of IL-1b (A), MMP-3 (B) and MMP-13 (C). Treatment with BIO-PLY resulted in significantly decreased synoviocyte *IL-1β* and *MMP-13*, but not *MMP-3*, expression levels compared to non-treated controls and equivalent to hyaluronic acid treatment (Hyvisc®) and triamcinolone acetonide corticosteroid (KENALOG®). (No Stim = Non-stimulated control; LPS Stim = LPS stimulation; No Trt = Non-treated control; HA = hyaluronic acid treatment (Hyvisc®); and Steroid = triamcinolone acetonide corticosteroid (KENALOG®). Bars are means and standard deviations of co-cultures from 6 horses and significant differences ($p<0.05$) between treatments are indicated by differing letters as determined by ANCOVA with Tukey post-hoc.)

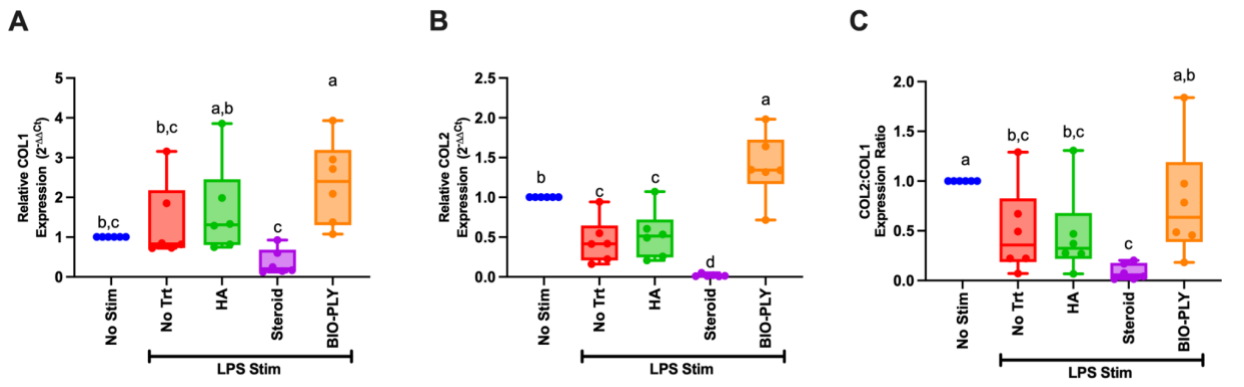


Figure 1.4. BIO-PLY significantly increases *COL1A1* and *COL2A1* gene expression without altering the *COL2A2*:*COL1A1* ratio. Chondrocyte gene expression levels of COL1A1 (A), COL2A2 (B) and resultant ratio of COL2A1:COL1A1 (C). Treatment with BIO-PLY resulted in significantly increased chondrocyte COL1A1 and COL2A1 expression levels compared to controls and other treatments without alteration of the COL2A2:COL1A1 ratio. (No Stim = Non-stimulated control; LPS Stim = LPS stimulation; No Trt = Non-treated control; HA = hyaluronic acid treatment (Hyvisc®); and Steroid = triamcinolone acetonide corticosteroid (KENALOG®). Bars are means and standard deviations of co-cultures from 6 horses and significant differences ($p<0.05$) between treatments are indicated by differing letters as determined by ANCOVA with Tukey post-hoc.)

The major roadblock associated with BIO-PLY is the in-house production of the product and requirement for donor horses. Parallel to the growing interest to characterize the cellular composition of various PRP preparations to identify the bioactive contents and most beneficial factors (28,34,80), the research team sought to isolate the antimicrobial peptides (AMPs) from BIO-PLY. This was based on their interest in the treatment of infectious OA as well as traditional OA. AMPs are often cationic innate defense regulator (IDR) peptides and can be synthetically

made to mimic naturally occurring innate immune effector molecules known as host defense peptides (81). The team utilized chromatography to separate and identify the bioactive peptide fractions within BIO-PLY and performed mass spectrometry on the identified fractions. Next, *de novo* sequencing was used to create a library of hundreds of peptide sequences that are undergoing selection by computer bioactivity model based on amino acid composition and predicted antimicrobial activity (82). Although the synthesis of these equine and BIO-PLY specific peptides is still underway, the lead peptides that have been flagged and the whole peptides of CXCL4, CXCL7, CCL5 and Tβ4 will serve as treatments in *Chapter 3*. In order to explore the use of host derived peptides from BIO-PLY, the four lead peptides will be tested to determine if they increase the synthesis of HA and decrease IL-1β and MMP-13 synthesis in equine synoviocytes in addition to the increase in collagen synthesis in chondrocytes.

1.3 | Conclusions

Osteoarthritis is a debilitating joint disease and can seriously affect large equine populations; therefore, finding a treatment that efficiently downregulates the catabolic matrix metalloproteinases and the pro-inflammatory cytokines without downregulating, and preferably while upregulating the important anabolic molecules such as hyaluronic acid, and collagen production. Platelet-rich plasma, its improved counterpart PRP-lysate and the further specialized product BIO-PLY have all demonstrated anti-inflammatory and chondroprotective effects within osteoarthritic joint environments both *in vivo* and *in vitro*. Not to mention, the inconsistencies within the available literature regarding clinical osteoarthritis therapeutics studies prompt a call to action to improve the methodologies utilized in order to provide credible and replicable results. Understanding the immense financial and institutional barriers associated with planning a clinical

trial accepted by the Food and Drugs Administration (FDA), it is important to investigate other novel options relating to the orthobiologic such as the peptide derivatives as allogeneic biologics face more challenges going through FDA processes. The research team has sought out a way to test peptides determined from the mass spectrometry of BIO-PLY as a synthetic alternative that could be used in the future as an "over-the-counter" therapeutic. Based on the current research discussed more in depth in *Chapter 3*, there is a gap in the academic conversation for how these lead peptides interact within stimulated synoviocytes and chondrocytes and what the synergistic effect of these might be. Therefore, there is a need for quality studies with efficient controls to provide data and evidence in both of these gaps to further the research progress in regenerative medicine.

CHAPTER 2

Drafting the Next Steps for Orthobiologic Research: A Future Clinical Trial

ABSTRACT

The gold standard for orthobiologic research are clinical trials to investigate the outcomes of novel treatments within patient populations. This chapter aims to review the broad literature of biological therapeutic clinical trials for the treatment of OA to discern the common shortcomings and limitations to be avoided in addition to using those themes as guidance to devise a clinical trial for a platelet-derived product in equine populations. The six major errors limiting the conclusions of clinical trials include (1) poor characterization of platelet-derived therapeutics, (2) lack of a placebo and/or inappropriate controls, (3) smaller-than-desired sample sizes, (4) short follow-up, (5) variability in case intensity and (6) inadequate parameters and grading systems. Therefore, the research team have designed the general framework of a prospective, double-blind, matched pairing, controlled randomized multicenter equine clinical trial complete with power analysis, inclusion and exclusion criteria, and grading and scoring systems. Future research teams can use this document as a translational blueprint when designing orthobiologic clinical trials with human, equine or other subjects in order to avoid the common pitfalls and yield the best data possible.

2.1 | Rationale

Platelet-rich plasma and its hemoderivatives continue to be used increasingly within both human and equine clinical settings with increasing research surrounding their effects (23–28). Numerous studies and considerable data have suggested the benefits of this orthobiologic, but the findings are inconsistent when different experimental and clinical trial results are compared (53). Several limitations of the experimental and clinical studies prevent the solidification of conclusions including the lack of consistency for PRP preparation as well as poor clinical trial design. Hence, there is a need for comprehensive clinical trials that mitigate the limitations of previous trials to produce more concrete outcomes to progress platelet-derived orthobiologics into application. Moreover, BIO-PLY, as a pooled PRP-lysate derivative, not only has promising *in vitro* results and has already undergone testing for safety *in vivo* with positive outcomes and no adverse effects noted; therefore, BIO-PLY is ready to begin clinical testing for the treatment of osteoarthritis.

The purpose of this chapter is twofold: (1) to review a selection of clinical studies in human and equine medicine examining the clinical effects of platelet-derived therapeutics and determine strengths and weaknesses of the studies and identify themes for possible areas of improvement when designing an orthobiologic clinical study, and (2) design a prospective, double-blind, case-matched, controlled randomized multicenter clinical trial in complete detail including overall framework, power calculations, inclusion and exclusion criteria, and grading systems.

2.2 | Thematic Review of Select Clinical Studies

Clinical studies serve as the highest standard of evidence for therapeutics and novel treatments in both human and equine medicine. However, recognizing the ethical and logistical considerations when undertaking a dynamic, multifaceted study like these, there are often unintended consequences which diminish the validity and credibility of results. Although not everything can be mitigated, there are proper precautions during the planning period prior to clinical rollout that can help prevent confounding variables and unclear conclusions from tainting meticulously collected data. Based on the selected clinical trials analyzed (Supplemental Table 2.1), systematic reviews and expert clinician opinions, there are six major errors impacting the results of a clinical trial: (1) poor characterization of platelet-derived therapeutics, (2) lack of a placebo and/or inappropriate controls, (3) smaller-than-desired sample sizes, (4) short follow-up, (5) variability in case intensity and (6) inadequate parameters and grading systems.

Debate continues to rage around the definition and classification for PRP and its related products because of the diverse cellular and cytokine profile associated with the various methods of preparation (54,55). Due to the incredibly dynamic nature of PRP from subject to subject, platelet concentration, growth factor, cytokine and leukocyte concentrations differs based on factors including age, sex, anti-inflammatory drug (NSAID) use, and for equine populations also includes the age, breed, time of day, hydration level, and exercise (41,56–58). This issue becomes a serious problem when ascertaining conclusions from clinical studies as the inconsistent preparations lead to inconsistent results (53). Although some studies analyzed with PRP as the treatment describe the therapeutic's preparation within the methods section, each style of preparation was different (46,50,72,83–88). In order to combat this, there is growing interest using pooled platelet-rich

plasma lysate (PRP-L) for bone and muscle regeneration (18,59–75). Some researchers have started testing PRP-L in human clinical trials (74). Nevertheless, PRP-L preparations also lack clear definitions and vary from study to study. Overall, although there is a wide use of PRP within veterinary medicine, some authors still don't adequately describe their PRP preparation, no valid standardized classification has been proposed and accepted, making PRP clinical studies incredibly difficult.

In a systematic review of equine PRP clinical trials, the reviewers found that not only were the majority of studies uncontrolled case series but also the largest study flaw was the lack of a true placebo control group (53). Of the 15 selected studies, 5 (33%) did not include a control group (50,74,83,88,89) and 6/9 (67%) of equine studies were either case series or *in vivo* experiments (72,85–89). Although these studies can serve an important purpose in determining the safety of novel treatments, those without control groups produce conclusions that are not definite and lack the ability to compare with treatments or the lack thereof. Ideally, a clinical experiment can have both a negative and positive control, such as equine osteoarthritis patients with no treatment, saline or sham (negative control) and subjects treated with the industry standard steroid injection (positive control). Through adequate controls, stronger comparisons can be made between treatment to provide more valuable data.

Sample size is an issue across the board in academia within all fields. Similarly in clinical trials, it is necessary to have a large enough sample to draw accurate conclusions that aren't biased. Across equine studies, sample sizes were smaller-than-desired, especially when compared to their counterpart human medicine studies (53). Studies having larger sample sizes ($n \geq 25$) had more

significant data (p values 0.05 to <0.001) and outcomes beyond efficacy or adverse reactions (72,89–92); meanwhile studies with extremely small sample sizes (n≤10) were much weaker despite statistical significance because they relied most heavily on performance outcomes like gait, return to sport and lameness (85,86,88). A power analysis with alpha at 0.05 and beta at 0.20 is required to determine the appropriate sample size between the expected outcomes for the treatment and control, with 10% additional buffer to account for dropout. These data used in the calculation should be based on the treatment's results *in vitro* when a new product is tested or based on previously established and verified clinical data. Multicentre studies included these larger sample sizes; therefore, collaborations with other hospitals or institutions may be a way to overcome sample size barriers.

The amount of time a study commits to follow-up data collection determines if the conclusions can be defined as short- or long-term. In all of the studies, except one human (93) and one equine study (89), the follow-up period did not expand beyond one year (46,50,72,74,83–88,91,92). As a result, the majority of these studies can not truly articulate the long-term effects or consequences of their platelet-derived treatment within osteoarthritic joints. Challenges such as extended funding, subject unenrollment, resources and labor all make consistent, long-term follow-ups difficult. Choosing adequate follow-up timepoints within the research team's capacity must align with the desired outcomes of a short- or long-term study. Furthermore, from the studies selected, an additional follow-up confounding variable existed as some had multiple PRP injections as a part of their treatment which may muddle data and conclusions more than before (46,50,72,74,84,87,93); yet, one human study disproved the relationship between a series of intra articular injections and more significant positive outcomes (46). If comparing PRP to the precursor

treatment triamcinolone acetonide, which is only done in one dose, then the therapeutic should mimic this pattern to reduce confounding variables.

Defining osteoarthritis clearly is present in all studies selected and from systematic reviews, but each article has different inclusion criteria for what OA means. For example, differentiating severe OA from mild or moderate OA through a clear definition is crucial especially if authors intend to distinguish the subjects they're studying from "average" cases (85). Often, studies group multiple grades together that require stratifications to separate the results between mild, moderate and severe osteoarthritis as comparing grade threes to grade ones would not provide accurate or meaningful data. Therefore, there are benefits from stratifications to separate grades or utilizing a matched pairs design.

Finally, inadequate parameters serve as the largest detriment to proper data collection to determine patient outcomes. Lameness, following AAEP guidelines, should serve as the primary data point as it is the clearest patient outcome indicator. Other scorings such as radiographic, joint effusion and ultrasound data points help to further confirm changes in disease progression or corroborate accurate osteoarthritis grading (72,85,86,88,89). If a study utilizes alternative scoring systems then they must be clearly defined and explicitly stated within the manuscript.

Acknowledging these six common shortcomings is salient in designing a clinical trial with the highest chance of success. These themes will be addressed in the following study design sections, with the first (3.3 | Overview) explaining how general study design combats these possible

limitations and the second (3.4 | Specific Parameters) contextualizing specific details that must be considered in each individual study through an example equine orthobiologic clinical trial.

2.3 | Study Design: Overview

There are hundreds of ways to design a clinical trial that most effectively delivers data to reach the project's aims and goals, yet the research team sees the most benefit in executing a prospective, double-blind, match pairing, controlled randomized multicenter clinical trial. This study design is optimized to mitigate the six possible limitations previously described as well as others to yield the highest quality of data.

Double-blinding the study helps reduce bias from being introduced by clients, scientists, technicians and clinicians; however, it would be naive not to mention the reluctance for clients to participate in the study if they are not sure if they are receiving the novel treatment being tested. Although the double-blind parameter is superior, a cross-over study could also be utilized to incentivize client recruitment by offering non respondent control subjects (those that did not receive the orthobiologic treatment) the opportunity to receive the tested treatment free of charge if there were no response after 3 or 6 months. Although this would prevent long-term comparisons in the case-matched design, it is more realistic as owners and clients might be hesitant to continue waiting if their musculoskeletal issues persist. On the other hand, introducing the cross-over design opens the door for new data collection to further supplement the initial study design, as the group originally treated as the control will have baseline values that can be analyzed additionally to the data collected after being given the investigated treatment. Nevertheless, the best possible setup

for blinding in these clinical trials is double-blinding but it's important to recognize the client hesitancy as a possible barrier and identify possible options to overcome them.

Moreover, matched pairing further ensures the analysis of representative data that accounts for differences in osteoarthritis severity using a collection of grading systems to create stratifications. Supplemental Tables 2.2, 2.3 and 2.4 detail grading systems for lameness, radiographic and joint effusion scoring that can serve as both baseline measurements in order to more effectively match control and treatment cases and as data collection methods throughout the study. When differentiating between mild and moderate osteoarthritis (with severe being placed in its own separate study), a general guideline involves using the The American Association of Equine Practitioners (AAEP) lameness grading system as the primary determinant of OA severity with radiographic, joint effusion, ultrasound or other axillary tests to pair cases with increased attention to detail. Leading with lameness as the primary data point allows for simpler pairing and processing because of the ease of evaluating horses under veterinarian guidance. Data would be compared both between the match pairs as well as from baseline to each time point to provide a full narrative for the changes in joint health.

Defining the study as a 'multicenter' clinical trial explains that the research team will compose of clinics across different sites to help with recruitment, enrollment and the delivery of the study. Recognizing the challenge of clinical trials on resources at a specific institution or clinic, collaborating with other researchers in the field helps distribute the burden rather than one team having to run everything. Sample size is a large predictor of the validity of findings and conclusions drawn from data with small sample sizes are often weaker than their large sample

counterparts; therefore, this framework helps increase the opportunity to reach the desired sample size as determined by a power calculation and recruit subjects in a faster time frame because of the different enrollment sources. As with all collaborations, clear protocols must be in place to prevent variation from site to site as well as explicit expectations in regards to manuscript contributions, authorship, etc.

2.4 | Study Design: Specific Parameters

The previous section provided general advice for study design regarding the large majority of orthobiologic clinical trials, whereas this section will focus on more specific details that must be considered for all studies but will be presented within the context of the BIO-PLY equine clinical trial. The purpose of this is to show how details of joint selection, data collection, inclusion and exclusion criteria, sample size, duration, controls, and safety look in application regarding the specific treatments, aims and goals of the study.

Selecting the joint of study is the first small detail in a study that has major effects on the conclusions drawn. Research teams should select a joint that is frequently/constantly used by the target population and is of interest and value for the development of novel treatments. For equine studies, selection of a specific joint can help limit variability rather than allowing a collection of different joints (unless the aim of the study is to compare across various joints). In our example clinical trial, we chose the fetlock joint, or metacarpo/tarsophalangeal joint (MCPJ/MTPJ) depending on if forelimbs (MCPJ) or hindlimbs (MTPJ), because of its high use, frequency within equine literature and efficacy to study as the most commonly affected joint with OA in the clinical setting. Other equine joints to consider include middle carpal joints (MC), antebrachiocarpal joints

(AC), carpometacarpal joints (CMC) and distal interphalangeal joints (DIJ). In human medicine, the most common joints studied for biological therapies are knee osteoarthritis cases followed by elbow and shoulder joints. Some studies have used contralateral joints as the baselines rather than matched pairs and even provided a sham treatment to further control for the injections. Joint selection will entirely depend on project goals.

Data collection is arguably the most important part of any study, let alone a clinical trial. Having multiple diverse data collection strategies allows for the presentation of stronger, more robust data and decreases the reliance on one set of data being usable. For osteoarthritis- and degenerative joint disease-based studies common grading techniques include radiographic data, ultrasound data, lameness analysis (AAEP grading for equine studies), lameness locator gait analysis, joint effusion and owner survey data. Relying only on owner survey data does not provide scientific or clinical evidence for treatment efficacy, but helps especially in studies with a goal for 'return to sport' outcomes. As mentioned previously, most of these data collection types have an element of subjectivity compared to something like a synovial fluid analysis; therefore, proper scoring should be done through a grading system with clearly defined severities with multiple details. Again, Supplemental Tables 2.2, 2.3 and 2.4 are example grading systems for lameness, radiographic and joint effusion scoring that would be used for the data collection of the example equine orthobiologic clinical trial because they provide a holistic look at the joint and horse as a whole when determining the efficacy of the pooled platelet-rich plasma lysate treatment BIO-PLY.

Defined inclusion and exclusion criteria are mandatory prior to enrollment in order to prevent confounding variables or unintended factors from altering the conclusions. For inclusion criteria,

the first should include some type of definition of clinical diagnosis for osteoarthritis or whichever degenerative joint disease is being studied along with the specific level of severity to be studied depending on project aims. For equine populations, the lameness should be located to the specific joint studied and should have complementary radiographic, ultrasonic or joint effusion evidence of joint disease. The research team has devised the following four statements for the inclusion criteria of the example study at the time of study enrollment:

1. Single limb lameness observable at the trot (AAEP lameness grade 2 or 3).
2. Lameness is localized to the fetlock joint via intra-articular analgesia (joint block).
3. Clinical evidence of osteoarthritis (enlarged joint with reduced range of joint motion accompanied by signs of pain) in that fetlock joint.
4. Radiographic evidence of osteoarthritis (osteophytes, subchondral bone changes, joint space narrowing, or irregular joint space) in that fetlock joint.

Moreover, exclusion criteria further defines what the subject population should not include to protect the homogeneity of the target population. Ensuring there aren't other major clinical issues that would complicate or affect the biological treatment studied are crucial and those subjects should be excluded. Subjects with osteoarthritis outside the range of investigation (too mild and too severe) and previous treatment in the short-term for their joint disease should also be excluded. Depending on the goals of the clinical trial, there may be other exclusionary criteria in order to refine the sample group to reflect the population being studied. The research team has devised the following exclusionary criteria for the enrollment of the example study:

1. Fracture
2. Active infection

3. History of chronic infection associated with the joint
4. Prior osteoarthritis treatments within 6 months of enrollment
5. Too mild (grades 0 and 1) or too severe (grades 4 and 5) osteoarthritis
6. Intra-articular injections or articular surgeries within 90 days
7. Any medications or orally administered supplements within 7 days

After determining the population to be studied in the trial to align with the project aims, a power calculation must be done to determine the required sample size. The standard values required for a power calculation include alpha (α) at 0.05, which is the probability of a type-I error or false positive rate, and beta (β) at 0.2, which is the risk of a type-II or a false negative rate. Determining the anticipated incidence or means for a novel orthobiologic treatment that has yet to be tested in a clinical model requires both creativity and data from *in vivo* experiments. Researchers should take data from the *in vitro* use of their novel biologic as well as the control they plan to use and apply those raw data points in the statistical power calculation to roughly establish the sample size. If the study employs a matched pairs design then the researchers must multiply the final value by two to ensure a 1:1 enrollment ratio and increase that product by 10% to account for subject withdrawal. In the power calculation example below, the research team used *in vitro* hyaluronic acid production data between the treatment, BIO-PLY, and the intended control, triamcinolone acetonide (TA). The average values of HA production from the experiment were 91,281 pg/mL for BIO-PLY and 53,473 ± 32,190 pg/mL for TA. For this study, the following mathematical process was used to determine the sample size of 26 horse subjects (13 per group) (mathematical symbol definitions found in Supplemental Table 2.5):

$$k = \frac{n2}{n1} = 1$$

$$n1 = \frac{(\sigma 1^2 + \sigma 2^2/K)(z1 - \alpha/2 + z1 - \beta)^2}{\Delta^2}$$

$$n1 = \frac{(32190^2 + 32190^2/1)(1.96 + 0.84)^2}{37808^2}$$

$$n1 = 11$$

$$n2 = n1 * K = 11$$

$$n1 + n2 = 22 * 1.10 \approx 26$$

$$n.total = 26$$

The final specific details to finish designing the clinical study include the treatment and control dosages, duration of the data collection and the safety of the trial. Whatever the control and treatment are, they must be standardized to each other and should have the same number of injections at equal increments (unless the study is investigating different treatment intervals and frequencies). In this study's case, triamcinolone acetonide is commonly a single injection in clinical practice at a dosage of 9 mg; therefore, the BIO-PLY treatment needs to be used as a single injection to match the control. Furthermore, orthobiologics are notorious for having dynamic properties depending on their donor, processing and more; so, it is critical for the continuity of the study for every injection used to be from the same lot and aliquoted equally with quantified protein levels and fractionation reports shared with the data. If each subject were to receive a different lot of biological treatment, it will diminish the credibility of the findings and could confound data. Duration of the study is dependent on aims, but follow-ups at regular intervals are crucial for determining efficacy of the treatment. Data collection points for both controls and treatments

should be more frequent in the beginning and can thin out over time, with long-term studies requiring a follow-up beyond 12 months, or one year. The example study intends to have data collection at months one, two, three, six and possibly 12 depending on if enrollment is consistent through one year. Understanding that most follow-ups require owners to bring their horse, or patients to get transportation to the clinic, more frequent follow-ups decrease the likelihood of enrollment and retention. Finally, safety is of utmost importance for these clinical trials and should be approved through proper IRB and IACUC protocols in addition to adequate *in vivo* safety testing prior to treating clients. BIO-PLY has been utilized in clinical settings and has proven to be safe when tested in equine musculoskeletal cases.

2.5 | Conclusions

To truly determine if a novel orthobiologic is effective as a treatment within a population, a clinical trial must be completed. Designing clinical trials can be difficult and overwhelming, but this article set out to breakdown the most common weaknesses of clinical biological studies and set forward a framework to use as a blueprint to define all necessary parameters in a clinical study.

There were six major errors reviewed from a selection of clinical trials, systematic reviews and expert clinician opinions along with suggestions to avoid their effects on data, which included:

1. Poor characterization of platelet-derived therapeutics is combated by using the same batch, lot and equal aliquots of the novel orthobiologics and documenting all protein analysis and fractionation.

2. Lack of a placebo and/or inappropriate controls can be avoided by having a proper negative control and/or positive control that is as similar in injection frequency as the biologic investigated.
3. Smaller-than-desired sample sizes are addressed through appropriate power calculations using mathematical models with *in vitro* data as the anticipated incidence or means if there is no clinical data available and by using a multicenter design to increase enrollment sites.
4. Short follow-up periods limit the ability to draw long-term conclusions; so if a research team wants to draw other conclusions the study should extend beyond one year (12 months) of follow-up.
5. Variability in case intensity is countered using clearly defined grading systems that are built into inclusion criteria and can also be used to match cases between the control and treatments to ensure joint disease severity doesn't confound results.
6. Parameters and grading systems must be adequately defined with clear stratifications based on national standards such as the AAEP in veterinary medicine and should be diverse by including at least two different types of data collection methods not including owner surveys.

Moreover, there are key parameters in the study design that must be adequately flushed out to ensure strong clinical trials. Some of these include:

- Deciding if the double-blind parameter is sufficient for the study population or if a cross-over study might be better.
- Determining the stratifications used if there will be a matched pairs element and how the pairs will be compared to ensure the most equal pairings possible for later analysis.

- Selecting a specific joint to study with predetermined, diverse scientific data collection methods to analyze the treatment efficacy in the isolated joint and in the organism as a whole.
- Explicitly stating inclusion and exclusion criteria prior to enrollment to define the degenerative joint disease and population to be studied and remove all possible confounding variables such as comorbid conditions or recent treatment.
- Ensuring safety of the treatment *in vivo* and proper IRB and/or IACUC approval before beginning enrollment and data collection.

Ultimately, the most important detail before outlining any clinical trial is to determine the aims and goals of the project because these will ultimately impact specific choices within the design process. Nevertheless, this didactic article can still serve as a guiding document in creating any orthobiologic trial.

CHAPTER 3

Preliminary viability and Nanostring nCounter™ analysis of BIO-PLY-derived lead peptides in cell monocultures show promise, prompt further discussion and investigation

ABSTRACT

BIO-PLY has proven itself to be a superior orthobiologic in *in vitro* lipopolysaccharide (LPS)-induced osteoarthritis (OA) experiments for synoviocytes and chondrocytes. Unfortunately, due to its allogeneic design as a pooled product, approval for clinical trials is a more rigorous challenge. Therefore, BIO-PLY-derived peptides, as determined by mass spectrometry and de novo sequencing, may serve as a synthetic alternative to create off-the-shelf disease-modifying therapeutic. Four of these peptide classes were selected, CXCL4, CXCL7, CCL5 and Tβ4, and were deemed as lead peptides based on their human availability and large frequency of equine specific peptides that fall under their motif. The purpose of this study was to explore the efficacy of these four peptides both in combination and in isolation as a treatment for LPS-induced OA monocultures of synoviocytes and chondrocytes. Our hypothesis was that the lead peptides would be synovio- and chondroprotective and exhibit synergistic effects such as anti-inflammatory and HA synthesis in synoviocytes, and collagen production in chondrocytes. Passage 1 (P1) synoviocytes and chondrocytes were stimulated with LPS for 24 h followed by no treatment or treatment with a steroid control (triamcinolone acetonide), all four peptides together or each peptide individually for 24 h. Cell count and viability was evaluated at the end of the treatment period and RNA was extracted and analyzed via Nanostring nCounter™ technology for gene expression of hyaluronic acid synthase 1-3 (*HAS1-3)*, collagen types I and II (*COL1-2*), interleukins 1β and 6 (*IL-1β* and *IL6*) and matrix metalloproteinases 3 and 13 (*MMP-3*, *-13*). Treatment with the lead peptides of stimulated synoviocytes preserved cell count and viability,

maintained *HAS2* gene expression and downregulated *IL-1β* expression to that of the steroid control. For LPS-stimulated chondrocytes, lead peptides downregulated *IL6* gene expression and maintained *COL2* gene expression. The results of this study support continued investigation into not only these lead peptides, but other lead peptides from the BIO-PLY peptide library and BIO-PLY-derived equine specific sequences for the treatment of osteoarthritis and call for further *in vitro* investigation with described limitations mitigated to draw more accurate conclusions for the effects of these peptides.

3.1 | Background

Osteoarthritis (OA) is a degenerative disease in the joint accounting for lameness in 60% of adult horses and stems from the inability of chondrocytes and synoviocytes to balance anabolic and catabolic activities within the extracellular matrix (1–4,7,10). This includes the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β) and tissue necrosis factor α (TNF-α) from synoviocytes which stimulate matrix degrading enzymes (3,6,11) as well as metalloproteinases (MMPs) and aggrecanases from chondrocytes amongst other catabolic enzymes to decrease type II collagen and aggrecan in the extracellular matrix, therefore decreasing cartilage (12,13). These catabolic secretions from both cell types influence the progression in a positive feedback system and contribute to the chronic nature of the disease (15,21,22). The majority of treatments for OA focus on the treatment of symptoms, as seen with corticosteroid and hyaluronic acid injections; therefore, there is increasing interest for biological treatments that are disease-modifying including platelet-rich plasma (PRP) and its derivatives (4).

Platelet-rich plasma (PRP) is the concentration of growth factors and peptides from autologous platelets and its therapeutic use has grown within veterinary medicine in the field of sports medicine as a treatment for musculoskeletal injuries and disorders (23–28). In the context of osteoarthritis, PRP has been shown to be anabolic, anti-catabolic, anti-inflammatory and antimicrobial because of the growth factors and peptides within the platelets (29–31). Recognizing the platelet releasate is the beneficial aspect of PRP, research explored a platelet-rich plasma lysate (PRP-L) as an alternative to improve composition and remove allogeneic issues (18,59–75). BIO-PLY is a patent-pending fractionate, pooled derivative of platelet-rich plasma lysate that is more concentrated with bioactive factors and has been shown to be chondroprotective, promote synovial

proliferative and provide anti-inflammatory benefits (27,41,79). As with all platelet-rich plasma and lysate derivatives, BIO-PLY is a biologic that requires blood donors, processing and storage before injection is possible.

Antimicrobial peptides, innate defense peptides and host-derived peptides are all being investigated for their individual effects on tissue repair in an effort to remove the production limitations of PRP. Unfortunately, the growth factors tested are done at concentrations different than those found in PRP and don't exhibit the synergistic effect that the combination of proteins and bioactive factors in PRP have, making the extrapolation of the data difficult (30,31). However, when tested in combination, the growth factors have been shown to exhibit synergistic effects on cartilage matrix synthesis (31).

An important study, Turner-Brannen et al., investigates a cationic innate defense regulator peptide in synovial fibroblasts and is an important milestone in the research for host-derived peptides as a therapy for various joint pathologies (81). Specifically, the research examined the IDR peptide, IDR-1002, in IL-1β-induced responses with fibroblast-like synoviocytes associated with the pathogenesis of inflammatory arthritis. The study concluded that the IDR-1002 peptide suppressed the production of IL-1β-induced MMP-3 and enhanced the production of IL-1RA through altering the expression of NF-κB and JNK pathways. Similarly, HA has been shown to bind to CD44 receptors to inhibit IL-1β expression to decrease MMP production (41,83–85); therefore, it can be theorized that therapies which target the signal transduction pathway involving NF-κB may be most beneficial.

To overcome this barrier, the research team has completed chromatography to separate and identify bioactive peptide fractions within BIO-PLY, and mass spectrometry on the identified fractions to create a library of peptide sequences to synthesize the beneficial peptides. *De novo* sequencing yielded a library of thousands of peptide sequences, but four lead peptides have been flagged as repeated parent proteins to the equine specific peptides. These lead, whole peptides include platelet-factor 4 (CXCL4/PF4), chemokine C-X-C motif ligand 7 (CXCL7/NAP-2), chemokine C-C motif ligand 5 (CCL5/RANTES) and Thymosin β4 (Tβ4) (94–98). These lead peptides have been observed within the osteoarthritic joint environment but have not been studied as a therapeutic to modulate growth factor healing effects. Therefore, there is a large gap in the academic conversation for how these peptides influence the cellular and extracellular composition within the joint.

Two of the main classes of chemokines secreted by platelets that constitute PRP-L and BIO-PLY including the C-X-C motif ligand (CXCL) and C-C motif ligand (CCL), and within these subtypes are major platelet chemokines including platelet factor 4 (PF4/CXCL4), CCL5 (or RANTES) and beta thromboglobulin (β-TG/CXCL7/NAP-2) (94–98). These chemokines, along with many others including thymosin β4 (Tβ4), have been well documented within synovial inflammation and OA with overwhelming evidence pointing to the chemokines as a reason for the progression in the disease process (99–102). Fibroblast-like synoviocytes and synovium tissue of patients with OA are shown to express CCL5 (103–107), which interacts with receptors in cartilage and may cause MMP-3 induction in chondrocytes (108–110). Several synthetic, non-peptide chemokine antagonists have been studied to reduce the severity of symptoms in mice, including the use of an antagonist of CXCR2 (the receptor of CXCL8/IL8 and CXCL7/NAP-2) which reduced the

neutrophil, monocyte and lymphocyte counts within the synovial fluid in a rabbit arthritis model and the use of a CCR5 antagonist (the receptor of CCL5/RANTES) reduced the severity of collagen induced arthritis (111–113). In summary, these lead peptides have been observed within the osteoarthritic joint environment but not as a therapeutic to modulate growth factor healing effects. There is a large gap in our knowledge of these four lead peptides and their influence within the joint.

This chapter aims to reveal preliminary data regarding which BIO-PLY-derived lead peptides, or a combination thereof, can be chondro- and synovioprotective *in vitro* within an osteoarthritic environment. The specific aims of this chapter are to (1) explore the proliferative effects of the lead peptides when used synergistically for both synoviocytes and chondrocytes monocultures, (2) in synoviocytes, examine the anabolic effects through the change in *HAS1-3* gene expression and catabolic effects through *IL-1β*, *MMP-3* and *-13* gene expression, and (3) in chondrocytes, examine the anabolic effects through the change in *COL1-2* gene expression and catabolic effects through *IL-1β* and *IL6* gene expression. We hypothesized that the lead peptides will be synovio- and chondroprotective and exhibit synergistic effects such as HA synthesis and anti-inflammatory in synoviocytes, and anti-inflammatory and collagen production in chondrocytes. Due to the novelty of this project, this article also serves to discuss the limitations and key takeaways to apply to future experiments for BIO-PLY-derived peptides.

3.2 | Methods

Study Design

A schematic of the study design is shown in Figure 3.1. The Institutional Animal Care and Use Committee of North Carolina State University approved the use of horses in these studies.

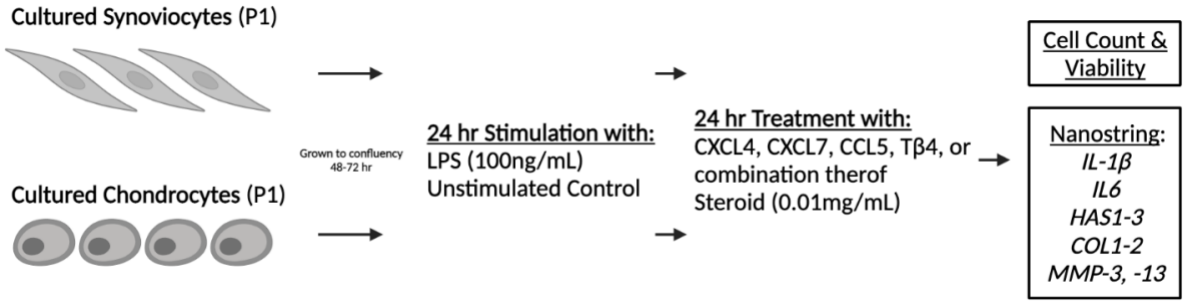


Figure 3.1. Schematic of study design.

Synoviocyte Isolation

Synovium was harvested and isolated from the femoropatellar joints of 5 healthy horses free of joint disease and euthanized for reasons other than this study. The isolated synovium was processed in synoviocyte media [high glucose (4.5 g/L) DMEM medium with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 1 mM sodium pyruvate, 25 mM HEPES, penicillin (100 units/mL), and streptomycin (100µg/ml)] containing 1.5 mg/mL and GibcoR collagenase type II (ThermoFisher Scientific, Waltham, MA, USA) (27). The digest was filtered at 100µm and centrifuged at 800 g for 10 min. The resulting cell pellet was washed with fresh media and synoviocyte cell count was determined using a CellometerR Auto 2000 and ViaStainTM AOPI Staining Solution (Nexcelom Bioscience LLC, Lawrence, MA, USA). Synoviocytes were frozen in aliquots of 10×10^6 cells/mL in liquid nitrogen until use.

Chondrocyte Isolation

Cartilage was harvested and isolated from the trochlear ridges of 5 healthy horses free of orthopedic disease and euthanized for reasons other than this study. The isolated cartilage was processed in chondrocyte media [Ham's F12 medium with 10% FBS, 25 mM HEPES, ascorbic acid (50µg/mL), α-ketoglutarate (30µg/mL), L-glutamine (300µg/mL), penicillin (100 units/mL), and streptomycin (100µg/ml)] containing 0.75 mg/mL and GibcoR collagenase type II (ThermoFisher Scientific, Waltham, MA, USA) (27). The digest was filtered at 100µm and centrifuged at 800 g for 10 min. The resulting cell pellet was washed with fresh media, resuspended and the chondrocyte cell count was determined using a CellometerR Auto 2000 and ViaStainTM AOPI Staining Solution (Nexcelom Bioscience LLC, Lawrence, MA, USA). Chondrocytes were frozen in aliquots of 10 × 106 cells/mL in liquid nitrogen until use.

Synoviocyte and Chondrocyte Seeding and Passaging

Cryopreserved synoviocytes and chondrocytes were thawed in a 37°C water bath then seeded in a 75cm^2 collagen I flask (ThermoFisher Scientific, Waltham, MA, USA) at 0.7 x 10^6 cells/flask in synoviocyte or chondrocyte growth media (114), respectively, and maintained at 5% CO_2, 90% humidity and 37°C. Cells were brought to confluency over 168 h (7 days) washing with DPBS (Corning Inc., Corning, NY, USA) feeding with fresh growth media after 24 hours from initial seeding and every 48 hours thereafter. Once cells reached a confluency of 2.8 x 10^6 cells/flask, the cells were passaged by incubating for 10 minutes with 0.25% Trypsin, 2.21 mM EDTA (Corning Inc., Corning, NY, USA) and cell count was determined using a CellometerR Auto 2000 and ViaStainTM AOPI Staining Solution (Nexcelom Bioscience LLC, Lawrence, MA, USA) before reseeding the passage one (P1) cells onto a 24-well, collagen I coated plate

(ThermoFisher Scientific, Waltham, MA, USA) and growth conditions were maintained. Cells were brought to confluency over 48 - 72 h before final media exchange prior to stimulation.

Synoviocyte and Chondrocyte Stimulation and Treatment

Cells were stimulated with E. Coli O55:B5 LPS at 100 ng/mL (Sigma-Aldrich, St. Louis, MO, USA) (55, 56). Unstimulated control wells underwent media exchange only. After 24 h of stimulation, select samples received a 0.01 mg/mL triamcinolone acetonide steroid control (Kenalog®, Amneal Biosciences, Bridgewater Township, NJ, USA) or an independent lead peptide (CXCL7, CXCL4, CCL5 or Tβ4) (Gen Script, Piscataway, NJ, USA) or a combination of all four. The peptide concentrations were calculated using previously published data to determine the number of platelets present in PRP and BIO-PLY products (27,115) and the amount of each specific peptide release from a single platelet (94,97,113,116–121) in order to provide physiologically accurate concentrations and conditions. After 24 h treatment, the media was pulled from each sample and stored at -20°C until use for ELISAs and the cells were immediately taken through RNA extraction protocol.

RNA Extraction

Total cellular RNA was extracted from chondrocytes using the RNeasy Mini Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions. The RNA purity and quantity were evaluated using UV microspectrophotometry (NanoDrop 2000 Spectrophotometer, ThermoFisher Scientific, Waltham, MA, USA). The concentration of the extracted RNA was 149.3 ± 42.1 ng/μL (range 232.5 - 60.3 ng/μL). RNA was stored at −20◦C until ready for gene expression analysis.

NanoString nCounter™

Digital multiplexed gene expression analysis was done using the NanoString nCounter™ System (NanoString, Seattle, WA, USA) according to the manufacturer's instructions (122). A custom NanoString CodeSet was developed to measure expression of critical genes associated with inflammation, chondroprotection, extracellular matrix synthesis and degradation in osteoarthritis (123). Anabolic genes assessed were: collagen (COL1A1, COL1A2, COL2A1, COL3A1) and hyaluronic synthase 1, 2 and 3 (HAS1-3). Catabolic genes assessed were: matrix metalloproteinases 3 and 13 (MMP-3, -13), interleukin 1-beta (IL-1β) and interleukin 6 (IL-6). Gene-specific probes designed by NanoString consisting of two half sites with 50bps included a target-specific capture probe biotinylated to attach to the cartridge and a target-specific reporter probe with a unique barcode for each transcript for imaging. The gene-specific probes and their accession numbers are listed in Supplemental Table 3.1. Total RNA (100 ng per sample) was hybridized using the nCounter™ GX CodeSet RNA Hybridization Setup instructions and incubated in a thermocycler at 65°C with a heated lid at 70°C for 18 hours, then set at 4°C until next step for a maximum of 12 hours (122). After hybridization, samples were placed in cartridges on the nCounter™ Prep Station (NanoString, Seattle, WA, USA) for purification. During the automated steps, excess probes were removed to remove background noise, hybridized probes bound to the cartridge in a vertical position and then an electric current allowed the probes to lie flat for more efficient imaging in the final step (122). Cartridges were moved to the nCounter™ Digital Analyzer for imaging and data collection. Unstimulated, non-treated cells were used as sample controls. All samples passed the quality control, positive controls demonstrated technical efficiency and data were normalized using the geometric mean of counts for the three reference genes beta-actin (ACTB), hypoxanthine

phosphoribosyltransferase 1 (HPRT1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

Normality was assessed for all results using the Shapiro-Wilk test. Normally distributed data was analyzed by the analysis of covariance (ANCOVA) with horse as covariate, followed by the Tukey's test for multiple comparisons. Non-normally distributed data was analyzed by the non-parametric Wilcoxon rank sum test. Statistical analyses were performed within the non-treated group across stimulations to assess the effects of stimulation and then within each stimulation group to assess treatment effects. Cell count analyses were performed using JMP Pro16 (SAS Institute Inc., Cary, NC, USA). Gene expression data were analyzed with nSolver™ 4.0 Analysis Software according to the user manual. Graphs were generated with GraphPad Prism 9 (GraphPad, La Jolla, CA, USA). Significance was set at $p < 0.05$

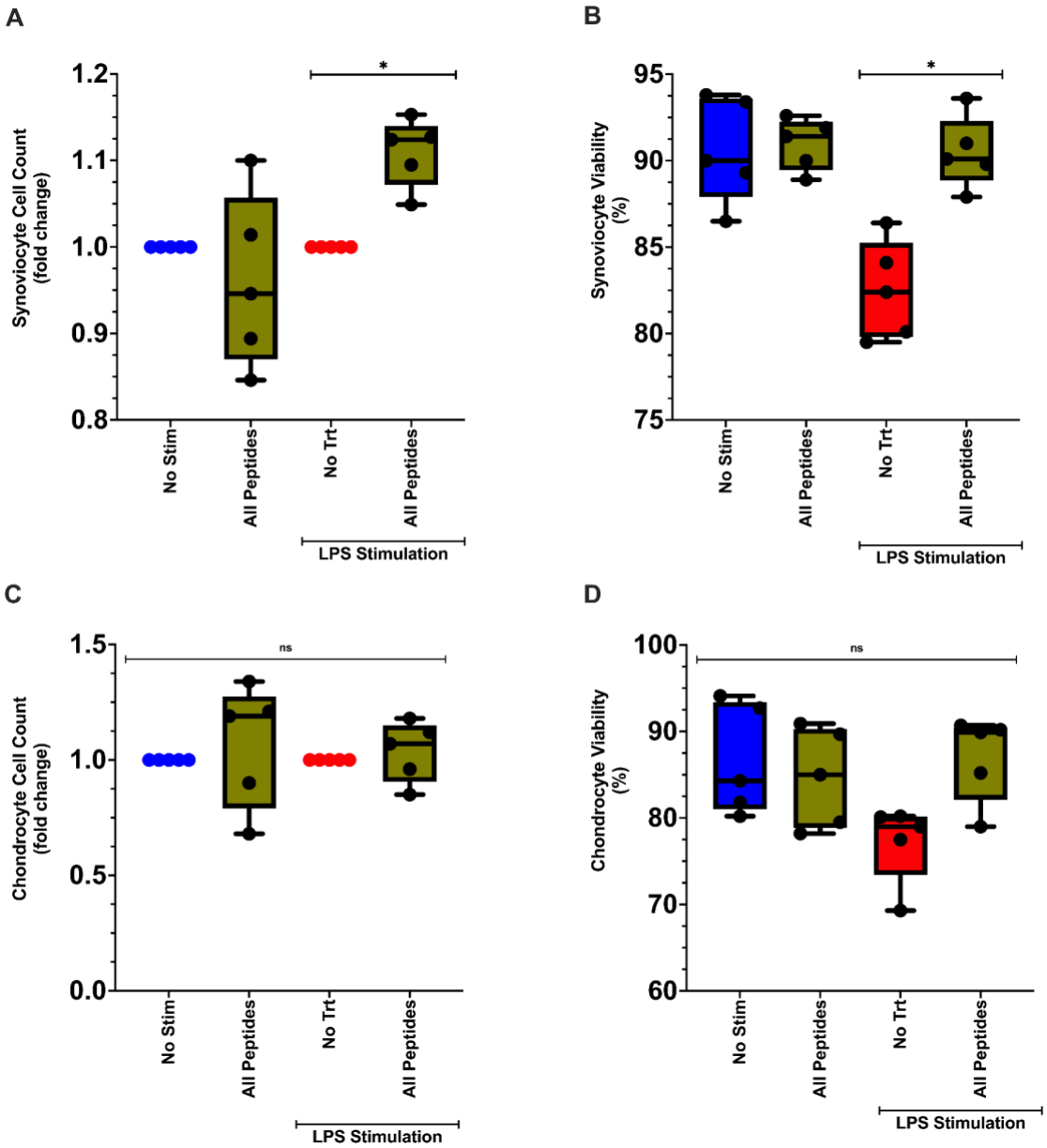
3.3 | Results

Lead peptides preserve synoviocyte cell count and viability.

When synoviocytes were unstimulated and treated with all four BIO-PLY-derived lead peptides (CXCL4, CXCL7, CCL5, Tβ4), there were no significant differences in cell count and viability (Fig 3.2A,B). However, when synoviocytes were stimulated with LPS and treated with all lead peptides, the mean fold change was 1.110 (SD = 0.03962) and was statistically significant compared to the LPS stimulated, untreated synoviocytes (p=0.0119) (Fig. 3.2A). The viability for stimulated, treated synoviocytes was significantly larger than stimulated, untreated synoviocytes (p=0.0202), and the difference in mean viability between the two groups was 7.98% (No trt = 82.50%, SD = 2.852; All peptides = 90.48%, SD = 2.079) (Fig. 3.2B).

Chondrocytes exhibited no significant difference in cell count and viability across both between unstimulated and stimulated groups and between untreated and treated samples (Fig. 3.2C, D).

Figure 3.2. Lead peptides, in combination, preserve synoviocyte cell count and viability, but not for chondrocytes. Equine synoviocytes (A-B) and chondrocytes (C-D) were left unstimulated (No stim) or stimulated with 100 ng/mL of LPS 24 h before being non-treated (No trt) or treated with all four BIO-PLY-derived lead peptides (CXCL4, CXCL7, CCL5 and Tβ4) at physiological concentrations for 24 h. Synoviocyte and chondrocyte cell count fold change and viability were measured via CellometerR Auto 2000 and ViaStainTM AOPI Staining Solution. Data are shown as the mean, with all individual values from the minimum to maximum of n = 5 for both cell types. There is no significant change in synoviocyte cell counts and viability between unstimulated controls and unstimulated, treated groups (A-B), but cell count (A) and viability (B) is significantly higher for synoviocytes stimulated with LPS and treated with the peptides. There are no significant differences for chondrocytes between stimulation and treatment for both cell count and viability (C-D). (No stim = no LPS stimulation, no treatment, No trt = LPS stimulation, no treatment, All Peptides = all four peptides (CXCL4, CXCL7, CCL5, Tβ4) at physiological concentrations. A single star (*) indicates significant differences between groups ($p < 0.05$) as determined from ANCOVA; statistical analysis was performed between treatment groups and their respective unstimulated or stimulated control.)


A
Synoviocyte Cell Count (fold change)
1.2
1.1
1.0
0.9
0.8
*
No Stim
All Peptides
No Trt
All Peptides
LPS Stimulation
B
Synoviocyte Viability (%)
95
90
85
80
75
*
No Stim
All Peptides
No Trt
All Peptides
LPS Stimulation
C
Chondrocyte Cell Count (fold change)
1.5
1.0
0.5
0.0
ns
No Stim
All Peptides
No Trt
All Peptides
LPS Stimulation
D
Chondrocyte Viability (%)
100
90
80
70
60
ns
No Stim
All Peptides
No Trt
All Peptides
LPS Stimulation

Some lead peptides maintain synoviocyte *HAS2* gene expression levels.

Synoviocytes stimulated with LPS and treated with a steroid control, all peptides together and each one separately showed no difference in *HAS1* and *HAS3* gene expression levels (Fig. 3.3A, C). *HAS2* gene expression differed significantly between the unstimulated samples (1 fold, SD = 0) and the LPS stimulated sample (1.856 fold, SD = 0.5550) ($p=0.0349$). All peptides together, CCL5 and Tβ4 were not statistically different from the unstimulated and steroid control samples ($p>0.05$); however, CXCL4 (2.06 fold, SD = 0.5495) and CXCL7 (1.834 fold, SD = 0.5366) were both statistically significant in maintaining slightly elevated *HAS2* gene expression exhibited when stimulated compared to the steroid treatment ($p=0.0068$ and $p=0.0399$, respectively) (Fig. 3.3B).

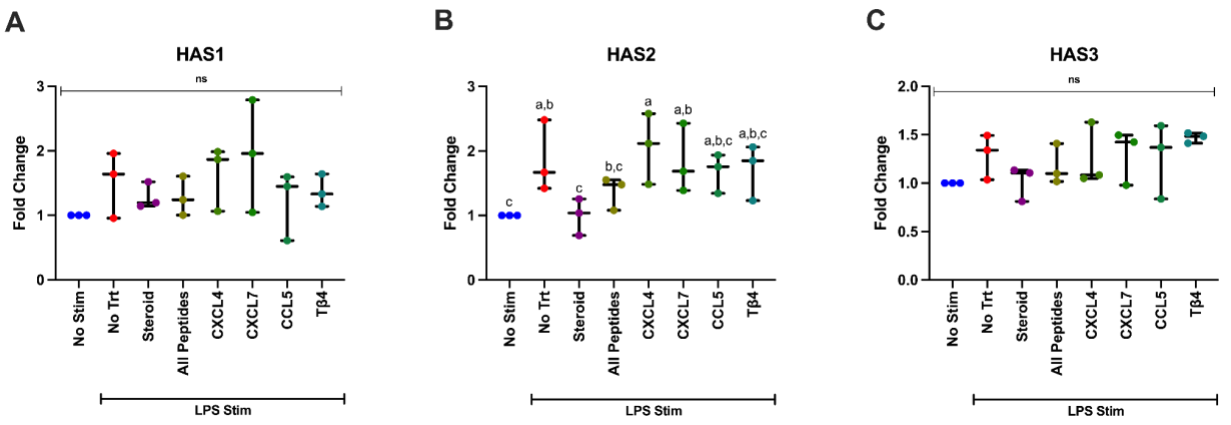


Figure 3.3. CXCL4 and CXCL7, in isolation, maintain synoviocyte *HAS2* gene expression compared to steroid but no treatments alter *HAS1* or *HAS3* gene expression. Synoviocyte gene expression levels of *HAS1* (A), *HAS2* (B) and *HAS3* (C). Treatment with specific BIO-PLY-derived-peptides CXCL4 and CXCL7 resulted in the maintenance of *HAS2* gene expression levels compared to the steroid control (B). There were no significant differences between any of the treatment groups for *HAS1* (A) and *HAS3* (C) gene expression. (No stim = no LPS stimulation, no treatment, No trt = LPS stimulation, no treatment, Steroid = triamcinolone acetonide corticosteroid (KENALOG®), All Peptides = all four peptides (CXCL4, CXCL7, CCL5, Tβ4) at physiological concentrations. Bars are the means of synoviocyte monocultures from 3 horses and significant differences ($p<0.05$) between treatments are indicated by differing letters as determined by ANCOVA with Tukey post-hoc.)

Treatment with all four lead peptides exhibits an anti-inflammatory effect.

All four lead peptides exhibit slight anti-inflammatory effects in both synoviocytes and chondrocytes. In synoviocytes, LPS stimulation increased *IL-1β* gene expression (2.978 fold, SD = 0.2214) whereas steroid control (0.9749 fold, SD = 0.3439) and and all peptides together (1.666 fold, SD = 0.3912) maintained *IL-1β* gene expression, with no significant difference

compared to the control ($p>0.05$) (Fig 3.4A). *IL-1β* gene expression was significantly reduced compared to the LPS stimulated, untreated sample for the steroid treated cells ($p=0.0041$) and all peptides treatment ($p=0.0128$) (Fig 3.4A). There were no significant differences in synoviocyte *MMP-3* and *-13* gene expression for any of the peptide treatments and the controls (Fig 3.4B, C). In chondrocytes, there was no significant decrease in *IL-1β* gene expression in any of the treatments compared to the LPS stimulated, untreated cells (Fig. 3.5A). However, there was a significant decrease in the *IL6* gene expression in samples treated with all four peptides (2.146 fold, SD = 0.6539) compared to the LPS stimulated, untreated cells (3.883 fold, SD = 1.083) ($p=0.0273$) (Fig. 3.5B). The downregulation is not statistically significant compared to the steroid treated control group (0.3682 fold, SD = 0.08645), although it trends towards being slightly higher ($p=0.0603$), and the unstimulated control group (1 fold, SD = 0) ($p=0.4643$).

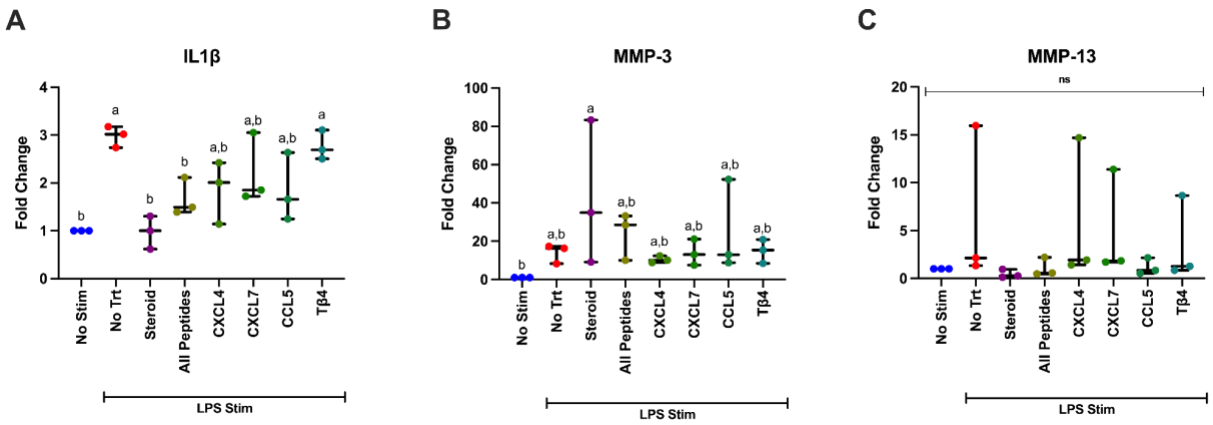


Figure 3.4. Treatment with all four lead peptides significantly downregulates synoviocyte IL-1β gene expression, but neither *MMP-3* nor *-13* gene expression. Synoviocyte gene expression levels of *IL-1β* (A), *MMP-3* (B) and *MMP-13* (C). Treatment with all four BIO-PLY-derived-peptides resulted in a significant decrease of *IL-1β* gene expression levels compared to the stimulated, no-treatment control (A). There were no significant differences between any of the peptide treatment groups for *MMP-3* (B) and *MMP-13* (C) gene expression. (No stim = no LPS stimulation, no treatment, No trt = LPS stimulation, no treatment, All Peptides = all four peptides (CXCL4, CXCL7, CCL5, Tβ4) at physiological concentrations. Bars are the means of synoviocyte monocultures from 3 horses and significant differences ($p<0.05$) between treatments are indicated by differing letters as determined by ANCOVA with Tukey post-hoc.)

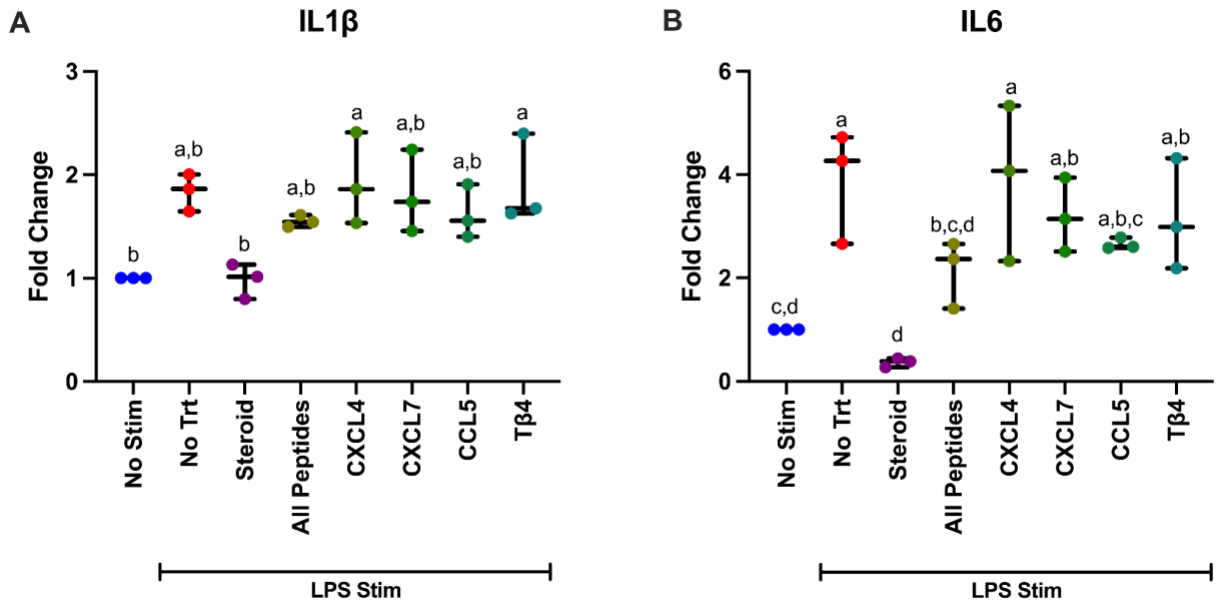


Figure 3.5. Treatment with all four lead peptides decreases chondrocyte *IL6* gene expression, but not *IL-1β* gene expression. Chondrocyte gene expression levels of *IL-1β* (A) and *IL6* (B). Treatment with all four BIO-PLY-derived-peptides resulted in a significant decrease of *IL6* gene expression levels compared to the stimulated, no-treatment control (B). There were no significant differences between any of the peptide treatment groups for *IL-1β* gene expression (A). (No stim = no LPS stimulation, no treatment, No trt = LPS stimulation, no treatment, All Peptides = all four peptides (CXCL4, CXCL7, CCL5, Tβ4) at physiological concentrations. Bars are the means of synoviocyte monocultures from 3 horses and significant differences ($p<0.05$) between treatments are indicated by differing letters as determined by ANCOVA with Tukey post-hoc.)

Some lead peptides maintain *COL2* gene expression levels in chondrocytes.

Chondrocytes stimulated with LPS and then treated with all four peptides in combination and isolation showed no significant change in *COL1* gene expression (Fig. 3.6A) compared with both

the LPS stimulated, no treatment control (0.8902 fold, SD = 0.2893) the steroid control (0.4894 fold, SD = 0.0592). For *COL2* (Fig. 3.6B), CXCL4 (1.318 fold, SD = 0.4161) and CXCL7 (1.088 fold, SD = 0.3627) maintained chondrocyte *COL2* gene expression levels close to unstimulated, untreated controls (1 fold, SD = 0) (p=0.7448, p=0.9998, respectively) compared to the steroid treatment (0.3273 fold, SD = 0.2306) (p=0.0037, p=0.0295, respectively). No other peptide-based treatments showed a significant difference between the controls and steroid treatment. The COL2:COL1 gene expression ratio showed no significant changes regardless of stimulation and treatment (Fig 3.6C).

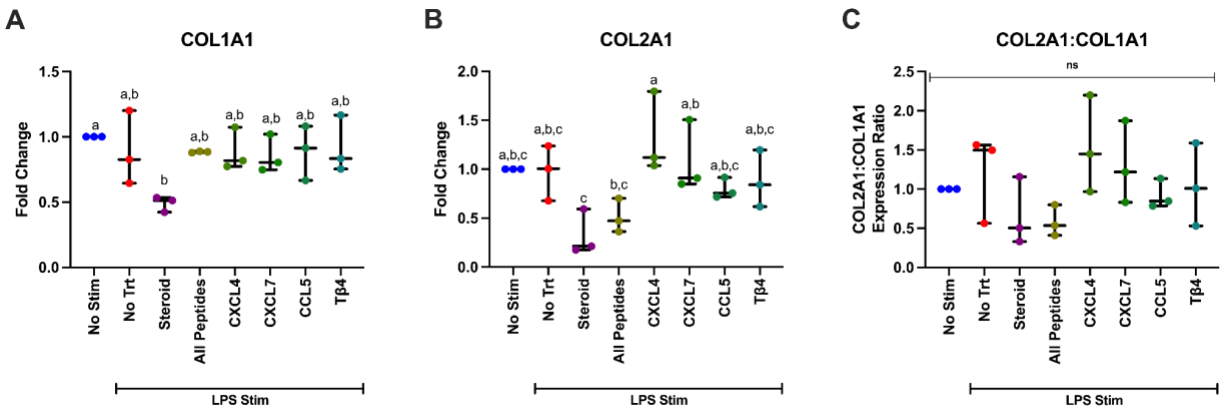


Figure 3.6. CXCL4 and CXCL7, in isolation, maintain chondrocyte *COL2* gene expression compared to the steroid, but not *COL1*. Chondrocyte gene expression levels of *COL1* (A) and *COL2* (B), and the COL2:COL1 ratio (C). Treatment with CXCL4 and CXCL7 alone maintained *COL2* gene expression levels compared to the steroid control, but no other treatments statistically differed from the steroid (B). No BIO-PLY-derived peptide treatments altered *COL1* gene expression levels (A) nor did any treatment group change the COL2:COL1 ratio (C). (No stim = no LPS stimulation, no treatment, No trt = LPS stimulation, no treatment, All Peptides = all four peptides (CXCL4, CXCL7, CCL5, Tβ4) at physiological concentrations. Bars are the means of synoviocyte monocultures from 3 horses and significant differences ($p<0.05$) between treatments are indicated by differing letters as determined by ANCOVA with Tukey post-hoc.)

3.4 | Discussion

In this preliminary analysis of BIO-PLY-derived lead peptides, some conclusions can be drawn from the reported findings despite limitations. Through the LPS model of stimulation for synoviocytes and chondrocytes to induce a similar response of acute OA, the BIO-PLY-derived lead peptides show promise as a possible therapeutic for equine osteoarthritis.

The selected lead peptides, CXCL4, CXCL7, CCL5 and Tβ4, have demonstrated a preservation of synoviocyte cell count and viability when treating previously stimulated synoviocytes, because the viability was maintained to the unstimulated controls whereas the stimulated, untreated synoviocytes experienced a significant decrease in viability. Describing the effect as prerving rather than proliferation is crucial, because there was no change in cell count between the unstimulated, untreated samples and the unstimulated, peptide treated group. Therefore, the peptides seem to have some protective effect on cell viability when synoviocytes are stimulated with LPS. Moreover, some of the lead peptides (CXCL4 and CXCL7) maintain the *HAS2* gene compared to the steroid control. Understanding that the triamcinolone acetonide corticosteroid will non-specifically downregulate both catabolic and anabolic genes, the steroid treatment significantly downregulates *HAS2* as well. Although the BIO-PLY data presented in *Chapter 1* shows BIO-PLY having a massive upregulating effect on synoviocytes, the lead peptides at least appear to not downregulate *HAS2* expression similar to steroids.

Arguably the most crucial aspect of these orthobiologics are their anti-inflammatory effects, seen very clearly with BIO-PLY and now seen slightly with these four peptides. In synoviocytes, there is a significant downregulation of *IL-1β* gene expression when all four peptides are used together that is comparable to the steroid control. This similar phenomenon is seen in chondrocytes with a significant downregulation of *IL6* with all four peptides in combination. Reducing the gene expression of both of these interleukins within the early stages of osteoarthritis is crucial to modify the disease progression out of the proinflammatory state it is headed towards. In looking ahead, it is critical to continue prioritizing the anti-inflammatory

effect of the treatment to yield a disease-modifying therapeutic rather than just a symptom-modifying treatment.

Finally, within chondrocytes there is a change in *COL2* gene expression noted within the short time frame between the steroid control and two peptides: CXCL4 and CXCL7. Similar to the pattern observed with synoviocytes and *HAS2* gene expression, *COL2* is downregulated by triamcinolone acetonide but the two peptides in isolation help maintain levels of *COL2* gene expression at unstimulated and untreated controls. Again, BIO-PLY drastically upregulates both *COL1* and *COL2*, but seeing the maintenance of *COL2* with these peptides does show promise for these peptides.

These conclusions prompt further investigation recognizing the possible benefit they can bring to the field of regenerative medicine to continue the academic conversation in disease-modifying therapies. Although finding a 'silver bullet' combination of peptides that can mimic the thousands of bioactive fractions and peptides in BIO-PLY is close to impossible, investigating these lead peptides along with the others identified from the mass spectrometry and de novo sequencing helps provide an alternative to the allogeneic orthobiologic that must go through more rigorous FDA approval compared to synthesized peptides. These experiments have shown more than inspiring preliminary findings, but also important limitations that must be addressed and mitigated as future studies explore platelet-derived peptides.

Similar to clinical studies discussed in *Chapter 2*, sample sizes are crucial in drawing strong conclusions about the efficacy of a treatment *in vitro*. In this study, the sample size was small

(n=3) for the Nanostring analysis because two outliers were excluded from the data due to a varied LPS response. This unfortunate exclusion of data from the gene expression analysis is due to the incredibly variable nature of LPS which can differ from lot to lot and have different effects from subject to subject. In an attempt to remove this limitation, exploring alternative *in vitro* OA models is crucial. IL-1β has been used previously as a stimulation technique, but a major limitation of using IL-1β is that the ng/mL range to obtain a desired response is far above the physiological pg/mL amounts (123). A new method using fibronectin fragments (Fn-f) is currently being investigated within the research group to help remove the variability created by LPS and be at a physiological concentration more common for OA (123). Hopefully the Fn-f model can be used to replace the LPS model to produce more consistent results and ensure a large enough sample size for accurate conclusions.

BIO-PLY was not used as one of the treatment controls for this study and therefore the research team relied on historical data to compare the effects of the lead peptides to BIO-PLY's effects. However, Nanostring nCounter™ gene analysis data is not comparable to qPCR $2^{-\Delta\Delta Ct}$ values; therefore, BIO-PLY must be used in tandem with other treatments using the transcript counting Nanostring methodology in order to accurately compare BIO-PLY-derived lead peptide efficacy to the original, parent biologic. As well, these peptides were not the exact BIO-PLY-derived peptides determined from de novo sequencing but rather the human versions of the parent peptide families that were common in the mass spectrometry.

Looking ahead at future experimental designs to investigate platelet-derived peptides as a treatment for osteoarthritis, there are many different directions researchers can take to fill the

large gap of knowledge on this topic. One example is to test more human versions of the lead peptides identified from BIO-PLY such as invariant tryptophan residue (WW) domain binding peptides, fibrinogen alpha chain and beta chain peptides, and more. Not to mention, synthesizing and testing some of the equine BIO-PLY-derived peptide sequences would provide valuable information about how the specific equine sequences interact with synoviocytes and chondrocytes, and even both in co-culture. If Nanostring nCounter™ technology is continued to be used, then researchers must use BIO-PLY as a treatment to include data to prevent the reliance on uncomparable historical data from qPCRs. Other methodologies should include protein quantification in media through the use of ELISAs for important molecules like IL-1β and hyaluronic acid, which can also be tested based on its viscosity to determine if the HA is of high or low molecular weight. Finally, exploring the Fn-f model as an option rather than the LPS or IL-1β stimulation models might provide more realistic *in vitro* data than before.

In conclusion, these four BIO-PLY-derived lead peptides are not a 'silver bullet' to fight against osteoarthritis; but, the preliminary data shows promise that these peptides could potentially serve as an alternative to the allogeneic orthobiologic in the future. The peptides exhibited minor preserving effects on synoviocyte viability, anti-inflammatory effects for both synoviocytes and chondrocytes, and anabolic effects for *HAS2* and *COL2* gene expression in synoviocytes and chondrocytes, respectively. Although there were some limitations from the study, these have been acknowledged with examples for mitigation in future studies.

3.5 | Funding

This work was supported by an AAEP Foundation Graduate Student Research Grant (Gilbertie), Park Scholarship Enrichment Grant (Jenio) and the Fund for Orthopedic Research in honor of Gus and Equine athletes (F.O.R.G.E; Schnabel).

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CHAPTER 4

Conclusions and Next Steps

The breadth of research and knowledge surrounding equine orthobiologics has grown exponentially over the past several decades and the recent creation of BIO-PLY has yielded an ideal therapeutic that reduces the limitation of other host-derived biologics. The future for BIO-PLY is bright with simultaneous plans to undergo a clinical trial to determine the clinical efficacy of BIO-PLY for the treatment of osteoarthritis as well as the exploration of BIO-PLY-derived peptides as a synthetically made option to reduce the barriers of FDA approval for allogeneic biologics.

This thesis set out to define osteoarthritis in the context of equine medicine and describe the history of platelet-rich plasma and its derivatives, design an optimal osteoarthritis clinical trial for BIO-PLY based on the themes from previous clinical studies in the field of orthobiologics, and gather preliminary data on some of the BIO-PLY-derived lead peptides identified and their effects on monocultures of synoviocytes and chondrocytes. *Chapter 2* outlined a prospective, double-blind, matched pairing, controlled randomized multicenter equine clinical trial complete with sample size calculations and justifications based on previous *in vitro* BIO-PLY data, detailed equine-based inclusion and exclusion criteria, and supplemental grading and scoring systems for lameness, radiographic scoring and joint effusions. *Chapter 3* concluded that the selected BIO-PLY-derived lead peptides (CXCL4, CXCL7, CCL5 and Tβ4) show promise as a possible therapeutic. The four selected peptides have shown a preservation of synoviocyte cell count and viability, some maintenance of synoviocyte *HAS2* gene expression (compared to

steroid), anti-inflammation effects for both synoviocytes and chondrocytes, and maintenance of *COL2* gene expression (compared to steroid). Nevertheless, due to the small sample size and exploratory nature of the study, these peptides prompt further investigation.

Moving forward, researchers can utilize the clinical trial described and the guidelines established in *Chapter 2* to design clinical trials for different biologics. This framework can continue to provide value beyond the first clinical trial completed with BIO-PLY as future clinical directions further investigate BIO-PLY efficacy in different contexts such as other musculoskeletal diseases and injuries, severe osteoarthritis cases, different species and more. As well, future scholars will be able to investigate platelet-derived peptides *in vitro* with all of the suggestions described in the discussion of *Chapter 3* and explore studies that analyze other platelet-derived peptides in monocultures, the four lead peptides selected in co-cultures, other stimulation models like fibronectin fragments and additional experiments like ELISAs to document protein concentration. No matter which direction researchers plan to go, the academic conversation will grow richer surrounding these biologics and their use within veterinary orthopedics and sports medicine and the translational applications to human medicine. Ultimately, this research can serve to find treatments that make not only our animal companions healthier, but also ourselves.

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APPENDICES

Supplemental Table 2.1 Review of human and equine clinical trials.

Supplemental Table 2.2 Lameness grading rubric

Supplemental Table 2.3 Radiographic scoring rubric

Supplemental Table 2.4 Fetlock joint effusion grading rubric

Supplemental Table 2.5 Mathematical symbol definitions

Supplemental Table 3.1 Nanostring nCounter™ Codeset Probes Design

Supplemental Table 2.1 Review of human and equine clinical trials.

Citation	Design	Treatment/ Control	Location	Scoring/Grading	Endpoints	Outcome	Conclusion(s)
Human							
(93)	Prospective, parallel-group, double-blind, multi-center, sham-controlled randomized clinical trial	P-PRP / Saline	Knee	WOMAC, IKDC subjective score, VAS score, intra-articular biochemical marker concentrations, cartilage volume, and adverse events	3, 6, 12, 24, 60 months	Only the P-PRP group showed a sustained improvement in clinical outcome measurements at month 24 ($P<0.001$).	At 6 months after injection, TNF-α and IL-1β levels in synovial fluid were lower in the P-PRP group ($P<0.001$).
(46)	Prospective, double-blind, randomized trial.	PRP / Saline	Knee	WOMAC, VAS, subjective satisfaction, Ahlback grade stratification	6 weeks, 3 months, and 6 months	Improvement in all WOMAC parameters was noted for PRP groups, with slight worsening at the 6-month follow-up ($P < .001$).	There was no difference between 1 injection vs 2 injections of PRP, and there was no influence of age, sex, weight, or body mass index on the outcome.
(83)	Prospective	PRP / None	Knee	VAS score and IKDC score, Kelllgren-Lawren grade stratification	1, 3, 6, 9, and 12 months	Later stages of OA reduced clinical effects of PRP ($P<0.05$) and increased the time for relapsed pain ($P<0.05$).	Increasing age and developing degeneration result in a decreased potential for PRP injection therapy.
(50)	Case series	PRP / None	Knee	IKDC score	6 and 12 months	All patients showed significant improvement in all	Both previously operated and non operated patients

						scores at 6 and 12 months (P < 0.01) and returned to previous activities.	showed significant improvement in symptoms and quality of life.
(84)	Prospective	PRP / HA	Knee	IKDC score, VAS score, adverse events and patient satisfaction	2 and 6 months	At 6 months' follow-up, better results were observed in the PRP group (P < .005).	Better results were seen within younger, more active patients with a low degree of cartilage degeneration compared to the later stage OA counterparts.
(74)	Prospective open label study	PL / None	Knee	KOOS score, Kellgren grades I and II	32 and 52 weeks	There was a significant improvement in the 5 aspects evaluated at weeks 32 and 52 compared with baseline (P < 0.0001).	Intra-articular PL significantly improved the score of all aspects evaluated by KOOS.
Equine							
(90)	Randomized, double-blind clinical trial	IA NASHA / Saline	MCP or MTP	SF samples, IA diagnostic anesthesia	2 weeks	No significant treatment effect for any biomarker was found between groups.	No clinical adverse effects were observed; concluded that IA NASHA was well tolerated.
(89)	Blinded clinical assessment, long-term	PAAG / None	MTP or one of the carpal joints	Lameness duration and grading, joint effusion grading, radiographic grading,	1, 3, 6, 12 and 24 months	There was a significant decrease in lameness grade from baseline to 1, 3, 6, 12 and 24	PAAG significantly alleviated lameness and

	follow-up			case details.		months ($P < 0.0001$) and a significant positive association with joint effusion ($P < 0.0001$).	joint effusion in osteoarthritic joints and could be an early OA treatment.
(91)	Prospective, randomized, parallel, open label, multicentre clinical trial.	HA / TA	Only one joint; DIP, MCP, MTP, MC or AC	Lameness and effusion scores, owner/veterinarian telephone questionnaire	3 weeks & 3 months	The success rate of IA TA 3 weeks after treatment was 87.8%, while that of TA+HA was 64.1% ($P = 0.01$).	The combination of TA with HA was associated with a lower short-term clinical success rate and a similar medium-term outcome compared with IA TA.
(92)	Prospective randomized masked placebo-controlled clinical trial	APS / Saline	Various high-motion joints (not elaborated)	AAEP lameness scale, kinetic gait analysis, joint signs of pain and swelling assessments, SF and blood analysis, client questionnaire	2, 12 and 52 weeks	The APS group had significant improvements in lameness grade ($P < 0.001$) by 14 days, compared with baseline or control group values.	IA administration of APS can be considered an effective treatment option for equine osteoarthritis, with the potential for disease-modifying effects.
(85)	Case-control/case series	PRP / Anesthesia	Fetlock or carpus joint	Radiographic grading, kinetic gait analysis	6 and 16 weeks	Out of 10 horses that responded to IAA, 3 responded to PRP at both time points and 4 responded at one.	Kinetic gait changes after intra-articular PRP are variable in horses with moderate to severe forelimb OA.

(86)	Experiment in vivo study/safety study	Thrombin-activated PRP / Saline	Fetlock or carpus joint	Synovial fluid analysis	6, 24, 48 and 96 h post-injection	Increased TGFβ1, TNFα and IL-6 in thrombin-activated PRP treated horses (P ⩾ 0.05).	Thrombin-activated PRP induced an inflammatory cytokine response in joints, whereas resting or CaCl2-activated PRP did not.
(72)	Case-control study	PL / Saline	DIP	AAEP lameness scale, radiographic grading	Fortnightly for a year	PL treated horses presented lower lameness grades ($p < 0.0005$) compared to controls 10 days after the second injection; radiographs revealed no changes in osteoarthritis lesions six months after treatment.	Majority of horses responded positively to PL treatment; however, all horses relapsed to their initial degree of lameness one year post-injections. IA PL is an efficient method for temporary management of OA.
(87)	Experimental control in vivo study	PRP / Saline	Randomly assigned MCP	Synovial fluid samples	3, 6, 24, 48, and at 168 h	Higher WBC and E2 prostaglandin in SF of PRP-treated joints ($P < 0.05$). No differences for IL-1β, IL-Ra, TNF-α, or HA concentrations between PRP and saline injected joints.	PRP injection elicits a mild and self-limiting inflammatory response shortly after administration, without long-term effects on joint homeostasis.

(88)	Case series	Autologous micro-fat and PRP / None	Fetlock or carpus joint	Radiographic grading, ultrasonographic grading, AAEP lameness score, return to competition	5 to 10 months	Nine joints were treated with significant improvement of the AAEP lameness score three months after the procedure ($p = 0.021$).	Four horses returned to official competition between 5 to 10 months after the procedure (7.0±2.5) and three of them resumed intensive training between 5 to 9 months (6.3±2.3).

Abbreviations (alphabetized): AAEP = American Association of Equine Practitioners, AC = antebrachiocarpal joint, APS - autologous protein solution, CMC = carpometacarpal joint, DIP = distal interphalangeal joint, HA = hyaluronic acid, IA = intra-articular, IKDC = International Knee Documentation Committee, KOOS = Knee Osteoarthritis and Disability Outcome Score, MC = middle carpal joint, MCP = metacarpophalangeal joint, MTP = metatarsophalangeal joint, NASHA = high-molecular-weight non-animal stabilized hyaluronic acid, PAAG = polyacrylamide hydrogel, PL = platelet lysate, PRP = platelet-rich plasma, SF = synovial fluid, TA = triamcinolone acetonide, VAS = visual analogue scale, WBC = white blood cells, WOMAC = Western Ontario and McMaster Universities Arthritis Index

Supplemental Table 2.2 Lameness grading rubric

Grade	Description	Eligibility
Grade 0	Lameness not perceptible under any circumstances.	Not eligible for enrollment
Grade 1	Lameness is difficult to observe and is not consistently apparent, regardless of circumstances (e.g. under saddle, circling, inclines, hard surface, etc.).	Not eligible for enrollment
Grade 2	Lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances (e.g. weight-carrying, circling, inclines, hard surface, etc.).	Eligible for enrollment
Grade 3	Lameness is consistently observable at a trot under all circumstances.	Eligible for enrollment
Grade 4	Lameness is obvious at a walk.	Not eligible for enrollment
Grade 5	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move.	Not eligible for enrollment
This grading scale is taken directly from the American Association of Equine Practitioners lameness grading guidelines.		

Supplemental Table 2.3 Radiographic scoring rubric

Grade	Description	Eligibility
Grade 0	No radiological findings of osteoarthritis.	Not eligible for enrollment
Grade 1	Doubtful narrowing of joint space and possible osteophytic lipping.	Eligible for enrollment
Grade 2	Definite osteocytes (<2 , small) and possible narrowing of joint space.	Eligible for enrollment
Grade 3	Moderate multiple osteocytes (2-4), definite narrowing of joint space, small pseudocystic areas with sclerotic walls, possible deformity of bone contour.	Eligible for enrollment
Grade 4	Large osteophytes or multiple osteocytes (>4), marked narrowing of the joint, severe sclerosis and definite deformity of bone contour.	Eligible for enrollment

Supplemental Table 2.4 Fetlock joint effusion grading rubric

Grade	Description	Eligibility
Grade 0	Concave appearance of the proximo-palmar recess of the MCP/MTP joint. No lateral swelling when medial pressure is applied to the recess.	Not eligible for enrollment
Grade 1	Flat appearance of the proximo-palmar recess of the MCP/MTP joint. Mild lateral swelling when medial pressure is applied to the recess.	Eligible for enrollment
Grade 2	Convex appearance of the proximo-palmar recess of the MCP/MTP joint. Lateral swelling is easily obtained when medial pressure is applied to the recess.	Eligible for enrollment
Grade 3	Convex appearance of the proximo-palmar recess of the MCP/MTP joint beyond the suspensory ligament branches (third interosseous muscle). Soft consistency of the recess on palpation.	Eligible for enrollment
Grade 4	Convex appearance of the proximo-palmar recess of the MCP/MTP joint beyond the suspensory ligament branches (third interosseous muscle) with a hard consistency of the recess on palpation, indicating synovial pressure. Synovial distension of the dorsal recess of the joint.	Eligible for enrollment
Grading guidelines from Bertoni et al, 2020.		

Supplemental Table 2.5 Mathematical symbols definitions

Symbols Definitions:
$\Delta = \lvert\mu 2 - \mu 1\rvert$ = absolute difference between two means
$\sigma 1, \sigma 2$ = variance of mean #1 and #2
n1 = sample size for group #1
n2 = sample size for group #2
n.total = total sample size for both groups
α = probability of type I error (0.05)
β = probability of type II error (0.20)
z = critical Z value for a given α or β
K = ratio of sample size for group #2 to group #1 (1 in a matched pairs design)
1.10 = accounting for 10% subject loss due to adherence

Supplemental Table 3.1 Nanostring nCounter™ Codeset Probes Design

Gene Name	Target Sequence
Beta-actin	CCCGGCCATGTACGTGGCCATCCAGGCCGTGCTGTCCCT GTACGCCTCTGGCCGCACCACTGGCATCGTGATGGACTC CGGTGACGGGGTCACCCACACT
COL1A1	CCCTGGCGAGCGTGGTGGACCTGGTGCCCGTGGCTTCCC TGGCGCAGATGGTGTTGCTGGTCCCAAGGGTCCCGCTGG TGAACGTGGTGCTCCTGGCCCT
COL2A1	AGAAACCATCAACGGTGGCTTCCACTTCAGCTATGGAG ATGACAACCTGGCTCCCAACACTGCCAACGTCCAGATG ACCTTCCTGCGTCTGCTGTCCACC
GAPDH	GACCAGGTTGTCTCCTGCGATTTTAACAGTGACACCCAC TCTTCCACCTTCGATGCTGGGGCTGGCATTGCCCTCAAC GACCACTTTGTCAAGCTCATTT
HAS2	CAAATCAGCCACTTACATCTGGAAGAACAACTTCCACG AGAAGGGTCCTGGTGAGACGGATGAGTCACATAAAGAA AGCTCTCAACATGTTACCCAATTG
HAS3	GGCTACCTATGCCTGCTTCCTTCGGGGCAATGCAGAGAT GATCTTCATGTCCCTCTACTCCCTTCTCTATATGTCCAGC CTCTTGCCAGCCAAGATCTTT
HPRT1	GTGTCATTAGTGAAACTGGAAAAGAAAAATACAAAGCC TACGATGAGAATTCAGGTTGAGTTGAGAAACATCTGGA GTCTCATTGAAATCACCAGTGAAA
IL-1β	GACTCCGGGACATATACCATAAATCCCTGGTGCTGTCCG GTGCATGTGAGCTGCAGGCTGTCCACCTCAATGGAGAG AATACAAACCAACAAGTGGTGTT
IL-6	GAAAACAACCTGAATCTTCCAAAGATGGCAGAAAAAGA CGGATGCTTCCAATCTGGGTTCAATCAGGAGACCTGCCT

	GATGAAAATCACCACTGGTCTTT
HAS1	GCTGCAAGCGCCAGGTCGTGTACGCCGCCTTCAAGGCG CTGGGCGCCGCCTTTGGAGTGAGAAAAACTCCCAGGCA TTTGGTAACCAGAGTACAGAAGTG
MMP3	AGCTCTGAAAATCTGGGAGGAGGTGACTCCACTTACATT CTCCAGGATTTATGAAGGAGAGGCTGACATAATGATCA CTTTTGCAGTTCGAGAACATGGA