

TURNING ROUND: OPTIMIZING THE ANTI-INFLAMMATORY PROPERTIES OF EQUINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS FOR OSTEOARTHRITIS THROUGH THREE-DIMENSIONAL CULTURE

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ABSTRACT

Osteoarthritis (OA) is a degenerative disease of diarthrodial joints causing pain and loss of joint function. Etiology is heterogeneous, but commonly involves inflammation arising from impairment of normal tissue homeostasis and/or function. A cycle of low-grade inflammation and global tissue degradation causes alteration of tissue morphology and function via primary mechanisms or inability to withstand physiological forces. Current therapies variably ameliorate symptoms but do not modify progression. Mesenchymal stem cells (MSCs) have multi-modal properties but are ineffective in ameliorating equine OA. However, anti-inflammatory activities of bone marrow derived MSCs (BMSCs) are enhanced by three-dimensional spheroid culture so equine BMSC (eBMSC) spheroids could inhibit intra-articular inflammation.

The overarching hypothesis is that eBMSCs can be enhanced to produce an allogeneic eBMSC therapy that inhibits intra-articular inflammation. *In vitro* experiments compared differences in anti-inflammatory phenotype between spheroid and traditionally cultured monolayer eBMSCs, the viability and health of eBMSC spheroids administered through needles, and the effects of allogeneic donor on the anti-inflammatory potential of eBMSC spheroids. A model of equine LPS induced synovitis was used to investigate anti-inflammatory efficacy of spheroid eBMSCs compared to placebo or monolayer eBMSCs *in vivo*.

eBMSCs aggregate into spheroids that have stable stem cell marker expression with increased secretion and gene expression of IL-6 and PGE₂, and gene expression of SDF-1 and TSG-6. IFN γ and TNF α were not produced by eBMSC spheroids and IL-10 production varied between individuals. Spheroids maintain higher viability and lower senescence than monolayer eBMSCs after injection through a needle and form in high-throughput culture without detrimental effects on expression of TSG-6, IL-6 and PGE synthases that denote an anti-inflammatory phenotype. Additionally, there is significant variation in this phenotype depending on the eBMSC donor.

eBMSC spheroids reduced total nucleated cell counts and objective lameness measurements at peak levels of intra-articular inflammation compared to monolayer cultured eBMSCs *in vivo*.

In summary, spheroids increase anti-inflammatory potential of eBMSCs and are practical for clinical use. Increased anti-inflammatory efficacy was demonstrated in a model of *in vivo* inflammation. This dissertation provides an understanding of the anti-inflammatory activities of eBMSC spheroids that can be used to develop an OA therapy.

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GENERAL AUDIENCE ABSTRACT

Osteoarthritis (OA) is a progressive disease of joints causing pain and loss of function. Multiple factors cause OA including inflammation, tissue destruction from enzymes, and breakdown due to reduced strength with continued use. This cycle of inflammation and joint tissue degradation causes joint tissue damage despite treatment with symptom relieving therapies. Mesenchymal stem cells (MSCs) are a multi-modal therapy, but have been ineffective to relieve equine OA. However, MSCs derived from bone marrow (BMSCs) have enhanced anti-inflammatory activity when produced by three-dimensional culture so BMSCs from horses could reduce joint inflammation better as three-dimensional spheroids.

The overarching goal of these studies was to produce an “off the shelf” horse BMSC therapy that reduces joint inflammation both for horse treatment, and as a model for human OA. These studies compared differences between spheroid and traditionally grown (monolayer) BMSCs to reduce inflammation, survival of spheroids administered through needles, and the variability between different horse donors on the ability of spheroids to reduce inflammation. The ability of spheroids to reduce joint inflammation was determined in live horses compared to control or monolayer BMSCs.

Horse BMSCs form spheroids that retain the properties that define stem cells, plus spheroid BMSCs produce factors that stem cells use to reduce the inflammatory response. Spheroids have enhanced survival compared with monolayer BMSCs after injection through a needle and spheroids can be produced in large quantities without affecting their potential to reduce inflammation. Additionally, BMSCs from different horse donors have varied potential to reduce inflammation. In live horses, donor horse BMSC spheroids reduced signs of joint inflammation and pain when inflammatory levels were highest compared to monolayer BMSCs. This dissertation demonstrates enhanced ability of spheroid BMSCs to reduce inflammation and provides key information that will be used to develop OA therapies.

Dedication

To my husband, best friend, budding stem cell culturist, groom, horse handler and housekeeper,
Daniel C. Vruink.

May we walk through a long and productive life together with our animals pain-free.

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Table of contents

Front Matter

Dedication	v
Acknowledgements.....	vi
Table of contents.....	vii
List of Figures	x
List of Tables.....	xii
List of Abbreviations.....	xiii
Overview of Dissertation	xv

Chapter 1 1

Introduction to Osteoarthritis: Pathophysiology and Therapy	1
The burden of osteoarthritis	1
Components of the diarthrodial joint in health and disease.....	3
Etiology of OA: Inflammation unites multiple inciting factors.....	13
Progression of OA: A cycle of inflammation and tissue degradation.....	17
Therapies for the treatment of OA.....	24
Summary.....	34

Chapter 2 35

Investigation and Role of Biologic Therapies for Osteoarthritis.....	35
The horse as a model for OA.....	35
Biological therapies for equine OA	38
Introduction to mesenchymal stem cells	42
Advantages and status of mesenchymal stem cells for osteoarthritis.....	50
Spheroid culture of mesenchymal stem cells to enhance therapeutic potential	58
Summary.....	62

Chapter 3 63

Three-dimensional Culture Enhances Anti-Inflammatory Potential of Equine Mesenchymal Stem Cells Regardless of Inflammatory Stimulation.....	63
Abstract.....	63
Introduction	63
Materials and methods	65
Results	70

Discussion	78
Conclusion.....	81
Chapter 4	82
Feasibility of Needle Injection and High-throughput Culture of Equine Bone Marrow	
Derived Stem Cells as Three-Dimensional Spheroids	82
Abstract.....	82
Introduction	82
Materials and methods	84
Results	87
Discussion	93
Conclusion.....	96
Chapter 5	98
Spheroids Cultured From Different Allogeneic Cell Lines Behave Differently in	
Inflammatory Conditions.....	98
Abstract.....	98
Introduction	98
Materials and methods	100
Results	103
Discussion	111
Conclusion.....	114
Chapter 6	115
Three-Dimensional Culture of Equine Bone Marrow Derived Mesenchymal Stem Cells for	
Treatment of Equine Synovitis.....	115
Abstract.....	115
Introduction	115
Materials and methods	118
Results	121
Discussion	132
Conclusion.....	136
Chapter 7	137
Conclusions and Future Directions	137
Summary of results	137

Future directions.....	139
References.....	141

List of Figures

Figure 1.1 – Ligament loading.....	8
Figure 1.2 – Subchondral bone histopathology	10
Figure 1.3 – Inflammatory events following joint injury	16
Figure 1.4 – Link between biochemical and gross pathogenesis of OA.....	24
Figure 2.1 – Stem cell types and differentiation potential	43
Figure 2.2 – Cytokine licensing of MSCs.....	48
Figure 3.1 –Spheroid formation.....	71
Figure 3.2 - DNA quantity	72
Figure 3.3 - Stem cell marker expression at 120 hours of culture monolayer vs spheroid culture.	73
Figure 3.4 – IL-10 and TSG-6 sph vs. mono	76
Figure 3.5 – IL-6, PGE ₂ , mPGES-1 and SDF-1 sph vs. mono	77
Figure 4.1 – Viability and senescence after needle passage	89
Fig. 4.2 – Monolayer colony forming units	89
Figure 4.3 – High-throughput spheroid aggregation.....	90
Figure 4.4 – Spheroid size as area and diameter length.....	91
Figure 4.5 – Size ranges of high-throughput culture	91
Figure 4.6 – Gene expression of anti-inflammatory phenotype markers by high-throughput culture	92
Figure 5.1 – IL-6 and TSG-6 by cell line.....	106
Figure 5.2 – mPGES-1 and PTGES2 by cell line	108
Figure 5.3 – IL-10 by cell line	110
Figure 6.1 - Goniometry for tibiotarsal joint flexion angle	120
Figure 6.2 - Linear clip mark location for sequential of joint circumference measurements	120

Figure 6.3 – Peripheral blood results	124
Figure 6.4 – Tibiotarsal goniometry results	125
Figure 6.5 – Tibiotarsal joint effusion results	126
Figure 6.6 – Inertial lameness results	129
Figure 6.6 – Synovial fluid total nucleated cell count	130
Figure 6.7 – Comparison of spheroid and monolayer TNCC	131

List of Tables

Table 2.1 - Published clinical trials using scaffold free intra-articular MSCs for the treatment of OA in humans	55
Table 2.2 - Published pre-clinical and clinical trials of scaffold free intra-articular MSCs for the treatment of OA in horses	56
Table 3.1 - Applied Biosystems Pre-designed Primer-Probe Sets.....	68
Table 3.2 - Custom Primer-probe sequences:	68
Table 3.1 - Visual summary of increase in immunomodulatory mediators compared to monolayer cells not treated with LPS (monolayer control/MC).....	79
Table 4.1 - Applied Biosystems Pre-designed Primer-Probe Sets.....	86
Table 4.2 - Custom Primer-probe sequences:	86
Table 4.3 - Area and diameter measurements $\pm$ S.E. for spheroids produced by Bioperfecta hanging drop or two types of low adherent flasks combined results from 3 horse cell lines.	88
Table 5.1 - Applied Biosystems Pre-designed Primer-Probe Sets.....	102
Table 5.2 - Custom Primer-probe sequences:	102

List of Abbreviations

2-D	Two-dimensional
3-D	Three-dimensional
AAEP	American Association of Equine Practitioners
ACS	Autologous conditioned serum
AdMSC	Adipose derived mesenchymal stem cell
ADMTs	A disintegrin and metalloproteinase with thrombospondin motifs
AGEs	Advanced glycation end products
BCS	Body condition score
BMI	Body mass index
BMSC	Bone marrow derived mesenchymal stem cell
COMP	Cartilage oligomeric matrix protein
COX-	Cyclooxygenase
COXIBS	Cyclooxygenase inhibitor
CS	Chondroitin sulfate
CSU	Colorado State University
CTX-II	C-telopeptide fragments of type II collagen
DAMPS	Damage associated molecular patterns
DMOAD	Disease-modifying osteoarthritic drug
eBMSC	Equine bone marrow derived mesenchymal stem cell
ECM	Extracellular matrix
ESCEO	European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases
EULAR	European league against Rheumatism
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
GH	Glucosamine HCL
GS	Glucosamine sulfate
HA	Hyaluronic acid
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon-gamma
IGF-1	Insulin-like growth factor 1
IL-	Interleukin-
IL-1ra	Interleukin 1 receptor antagonist
INAD	Investigational New Animal Drug
KL	Kellgren-Lawrence
LPS	Lipopolysaccharide
LRS	Lactated ringer's solution
MACI	Matrix-induced Autologous Chondrocyte Implantation
MCP-1	Monocyte chemoattractant protein-1
MHCII	Major histocompatibility complex class II
MMP-	Matrix metalloproteinase

Mono	Monolayer
MPA	Methylprednisolone acetate
mPGES-1	Microsomal prostaglandin E synthase-1
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
MTIII	Third metatarsal bone
NADA	New Animal Drug Application
NFκB	Nuclear Factor kappa B
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PDGF	Platelet derived growth factor
PGE₂	Prostaglandin E2
PRP	Platelet Rich Plasma
PRRs	Pattern recognition receptor
PSGAG	polysulfated glycosaminoglycan
PT-OA	Post-traumatic osteoarthritis
PTGES2	Prostaglandin E synthase 2
qPCR/ RT-qPCR	[Real-time] quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end-products
SASP	Senescence-associated secretory phenotype
SCB	Subchondral Bone
SDF-1	Stromal cell-derived factor 1
SF	Synovial fluid
Sph	Spheroid
SYSADOAs	Symptomatic slow-acting drugs
TA	Triamcinolone acetate
TGFβ	Transforming growth factor beta
TIMPS	Tissue inhibitors of metalloproteinase
TLRs	Toll-like receptor
TNCC	Total nucleated cell count
TNFα	Tumor necrosis factor alpha
TP	Total protein
TSG-6	Tumor necrosis factor-inducible gene 6
USDA	U.S. Department of Agriculture
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor
WBC	White blood cell count
WOMAC	Western Ontario & McMaster Universities Osteoarthritis Index

Overview of Dissertation

Chapter 1 investigates the clinical problem of osteoarthritis (OA) including epidemiology, pathophysiology and therapy. OA is a progressive, destructive and debilitating disease of diarthrodial joints characterized by a complex interplay of inflammation and tissue breakdown. Although researchers and clinicians have advanced the understanding of OA pathophysiology, therapies mainly improve symptoms rather than modify disease progression. As a result, both human and equine patients suffer from complete destruction of articular matrix, pain and loss of joint function. Given the impact of this disease, new treatments need to be developed that have both symptom relieving and disease modifying effects.

Biologic therapies represent a new frontier for symptom modification and tissue regeneration; the FDA [1] has recognized their development as safe and effective drugs. Chapter 2 investigates the current and potential roles that biologic therapies have in ameliorating OA and highlights the horse as a clinical model and end-stage recipient of these therapies. Mesenchymal stem cells (MSCs) represent a biologic with multi-modal activities, capable of anti-inflammatory, immunomodulatory and trophic functions. Their current use, limitations and potential advantages for OA are discussed and three-dimensional culture of MSCs is introduced as a technique to enhance their anti-inflammatory potential to ameliorate inflammation in OA.

MSC treatments have low efficacy to reduce clinical signs of equine OA and synovitis, but spheroid culture enhanced the anti-inflammatory potential of human bone marrow derived MSCs (BMSCs). Therefore, Chapter 3 aimed to investigate alterations in anti-inflammatory phenotype of equine BMSCs (eBMSCs) with spheroid culture. Through *in vitro* inflammatory stimulation of spheroids and traditionally cultured monolayer eBMSCs, we demonstrated that eBMSC spheroids produced higher levels of TSG-6, PGE₂, SDF-1 and IL-6 that play a role in inflammatory/immune modulation. eBMSC spheroids produced these mediators without exogenous inflammatory stimulation. Additionally, spheroids did not produce the inflammatory and immune-stimulatory cytokines TNF α and IFN γ , and had variable production of the anti-inflammatory cytokine IL-10. Chapter 3 raised questions of the variable IL-10 production between eBMSC donors and demonstrated that hanging drop spheroid production was time consuming and impractical to produce spheroids in sufficient quantity for clinical trials.

Chapter 4 aimed to answer the concerns of eBMSC spheroid practicality for clinical use by investigating viability and senescence of spheroids compared to monolayer culture when passed through 22g, 20g and 18g needles. In this chapter, we also developed a high-throughput method of eBMSC spheroid culture, making it possible to efficiently culture the 10-20 million eBMSCs normally used for equine intra-articular injection. Spheroids had enhanced viability and lower senescence compared to monolayer cultured eBMSCs, and high-throughput culture did not detrimentally affect the expression of TSG-6, IL-6 or PGE synthase, which have been found by other authors to be the key genetic markers of an anti-inflammatory BMSC phenotype [2-5]. In fact, high-throughput culture produced up-regulated the anti-inflammatory protein TSG-6, which is able to ameliorate activity of TNF α and IL-1 β [6].

Given the variability in production of anti-inflammatory cytokines by eBMSC spheroids and the enhanced ability for allogeneic eBMSC therapies to be readily available, Chapter 5 aimed to assess differences in the anti-inflammatory/immunomodulatory potential between spheroids cultured from different allogeneic donors. Significant variation between eBMSC cell lines in response to inflammatory stimulation with IL-1 β was discovered, and differences in expression of genetic markers of an anti-inflammatory phenotype varied in up-regulation depending on response to spheroid culture and/or inflammatory stimulation. This study aided selection of an allogeneic eBMSC line predicted to be appropriate for therapeutic use to inhibit equine joint inflammation.

Principles from Chapters 3, 4 and 5 were applied in the final experimental chapter that aimed to investigate the efficacy of spheroids to ameliorate inflammation in an *in vivo* model of equine synovitis, and differences in their efficacy compared to traditionally cultured monolayer eBMSCs. While spheroid culture did not ameliorate all signs of inflammation, it did have positive treatment effects on synovial fluid total nucleated cell count and lameness compared to placebo control or monolayer eBMSCs.

In Chapter 7 major conclusions, a general discussion and recommendations for future investigation of spheroid culture to ameliorate inflammation are discussed.

CHAPTER 1

Introduction to Osteoarthritis: Pathophysiology and Therapy

The burden of osteoarthritis

Osteoarthritis (OA) has most recently been defined by an OARSI-FDA initiative as a usually progressive disease of diarthrodial joints that represents failed repair of joint damage resulting from stresses arising in any of the synovial joint tissues ultimately resulting in the breakdown of cartilage and bone leading to pain, stiffness and functional disability [7]. The first published definition of osteoarthritis focused on the alterations in bone morphology seen on radiographs and included OA from a range of etiologies [8]. Although, the Kellgren-Lawrence grading scheme for radiographic signs of OA are still used today, it is widely accepted that defining OA by radiographic evaluation alone does not provide an adequate description of disease [7]. This is because the disease process is a whole organ disease, involving all tissues of the joint: articular cartilage, subchondral bone, synovium, joint capsule and ligaments [9]. Altman, et al. [10] recognized the heterogeneity of OA and defined OA as having different etiologies with similar morphologic and clinical outcomes. The most current definition of OA recognizes differences in both etiology and disease phenotype [7]. Therefore, current recommendations focus on understanding and treating specific OA subgroups rather than the disease process as a whole [7].

An estimate on the prevalence of OA in the USA in 2005 was 27 million people, which had increased since the estimate of 21 million in 1995 [11]. Accurate prevalence of OA depends on specific joints under investigation and characteristics of the population in question. For example, a different prevalence for knee OA in people was found in the Framingham study (27.8%) [12], compared to the Johnston County Osteoarthritis study (19.2%) [13] with discrepancies likely because the Framingham study was based in suburban Massachusetts and the Johnston County study was in rural North Carolina. Aged populations have a higher prevalence of OA with new cases of OA occurring at a rate of 2% and progression at a rate of 4% per year in people over 65 [12]. For horses, the prevalence of OA also varies with horse use, for example 54% of Thoroughbred racehorses admitted to a university hospital in the United Kingdom had evidence

of OA [14], but 10-19% of Quarter Horses used for Western Sports that were also admitted for lameness examination had OA [15,16]. Like humans, OA has increased prevalence with horse age, affecting 83.5% $\geq$ 15-year-olds [17].

OA incurs significant economic cost to both human health and the equine industry. In a study of 473 patients from Canada with rheumatoid arthritis, osteoarthritis, or high blood pressure; 70% had osteoarthritis that cost each patient on average \$4900-\$5700 US dollars in drug costs [18]. The total cost of OA and rheumatic disease treatment in the USA was \$128 billion in 2007 [19] and the economic burden of lost earnings due to absenteeism from osteoarthritis alone is \$10.3 billion dollars annually [20]. Likewise, the cost of lameness to the North American horse industry was estimated at \$1 billion per annum in 2001 [21] and is the leading cause of financial loss in Thoroughbred racehorses [22].

In particular, post-traumatic osteoarthritis (PT-OA) is of significant economic and social burden to both humans and horses. PT-OA is a sub-group of OA that develops as a result of trauma to any of the joint tissues. Human PT-OA patients are 10-15 years younger than primary OA patients, but incur equally debilitating disease and similar (\$11.79 billion) treatment costs despite comprising 12% of all OA patients [23]. Intra-articular injuries occur at a rate of 2% per month in Thoroughbred racehorses [24]. Such injuries contribute to a prevalence of up to a third of Thoroughbred racehorses that are affected by osteoarthritis by the time they are 2-3 years old [25]. Orthopedic injury, including osteoarthritis and intra-articular injury, accounts for a significant amount of wastage in the equine industry. Wastage is defined as any injury or disease that involves an interference with the training schedule of a horse, resulting in lost days in work, a prolonged spell or retirement from intended use [26]. It is well established that osteochondral chip fragments in the equine mid-carpal joint induce mild to moderate PT-OA in the Thoroughbred racehorse [27], and that horses with condylar fractures that involve the metacarpophalangeal articulation have decreased total earnings when they return to racing with prognosis related to the amount of intra-articular damage [28].

Components of the diarthrodial joint in health and disease

Overview of diarthrodial joint function

The diarthrodial or synovial joints provide friction free motion and flexibility to the appendicular skeleton, occurring at the junction between bones [29]. Diarthrodial joints are uniaxial, biaxial or multiaxial. Uniaxial joints move in one plane only, such as the femorotibial joint, cubital joint and interphalangeal joints. Biaxial joints involve an ellipsoid articulation allowing abduction, adduction or circumduction as well as flexion and extension, but they are not able to rotate. Examples include the temporomandibular joint and occipitoatlantal joint. Multiaxial joints are most commonly ball and socket joints and allow movement in all planes, for example the coxofemoral joint. Diarthrodial joints are complex structures that rely on interactions from multiple tissues with specific anatomical arrangement. Components of the diarthrodial joint are subchondral bone (SCB), calcified and articular cartilage, peri- and intra- articular ligaments, synovium and joint capsule and synovial fluid; together they act to transfer forces from the long bones applied by musculotendinous units and impact [29]. Dysfunction or injury to one or more structures alters the physical and physiologic homeostasis of the joint and can result in the process of OA that leads to eventual degeneration of all joint tissues.

Articular cartilage

Articular or hyaline cartilage aids friction free motion of diarthrodial joints as well as providing resistance against compressive forces. Mature articular cartilage is avascular, lacks nerves and lymphatics, and is composed of relatively few (1-12%) chondrocytes surrounded by extracellular matrix (ECM) [30]. As a result, articular cartilage relies on the synovial fluid for its nutrition. The ECM is composed of collagens, proteoglycans and water [31]. Articular cartilage is divided into four zones based on chondrocyte morphology and collagen orientation [32]. The superficial (tangential) zone is closest to the joint surface and has chondrocytes that are flat and, along with collagen fibers, are orientated parallel to the articular surface. In contrast, the intermediate (transitional) zone has larger ovoid to round chondrocytes and the deep (radiate zone) has large chondrocytes orientated perpendicular to the articular surface. The calcified zone is the deepest and has mineralized cells and matrix. It is distinguished from the non-calcified articular cartilage by a line called the tidemark that acts as an anchor point for collagen fibers.

Articular cartilage gains a highly organized structure as the animal grows. At birth, cartilage is immature with an isotropic structure and must develop into mature cartilage with anisotropic architecture [33,34]. Chondrocytes initially have a disorganized orientation within the matrix and collagen fibrils are randomly orientated. Chondrocytes gradually gain anisotropy by lining up in columns perpendicular to the cartilage surface, likewise collagen fibrils begin to orientate around chondrocytes to form distinct pericellular, territorial and extraterritorial regions of cartilage matrix [35]. The same study found that this process of matrix re-organization is through a process of resorption and formation in all cartilage zones. The characteristics of mature cartilage are completed by puberty, with no changes between puberty and skeletal maturity seen in New Zealand white rabbits [35]. Maturation of the articular cartilage contributes to longitudinal and surface growth of the epiphysis because superficial-zone stem cells divide both horizontally and vertically [33,35]. Vertically derived stem cells hypertrophy and later become mineralized in the deep zone. The mineralization front contributes to bone lengthening and thinning of the articular cartilage layer.

Chondrocytes produce and organize the cartilage matrix through the production of collagens, proteoglycans and hyaluronic acid. They have a high metabolic and replicative rate during growth, which decreases significantly in mature cartilage. Mature cartilage chondrocytes produce and remodel cartilage matrix components depending on their zonal organization as well as biological and mechanical cues from the surrounding matrix. For example, chondrocytes in the superficial zone produce less proteoglycans than those in the deeper layers, but more type II collagen [36]. Loading of the articular cartilage causes alterations in intracellular volume, pH and ions [37] that translates to electrical and physiochemical signals to regulate production of matrix components [38]. Loading at physiologic levels has been found to increase the proteoglycan and collagen content of articular cartilage matrix and immobility to reduce them [39,40]. However, excessive loading can also cause reductions in proteoglycans and catabolism of the collagen matrix [41,42]. In this way the articular cartilage matrix and chondrocytes intimately rely on each other to maintain cartilage homeostasis.

Chondrocytes not only respond to alterations in the nearby matrix, but also to alterations in all of the joint tissues through cytokine driven metabolic changes. Cytokines are peptides that are produced and released into the matrix or synovial fluid by neighboring chondrocytes,

synoviocytes or osteocytes and function in an autocrine or paracrine manner [43]. The cytokines commonly active in the joint environment are interleukins, interferons, tumor necrosis factors and growth factors. During physiologic remodeling low levels of IL-1 β induce chondrocytes to produce matrix metalloproteinases that are able to cleave collagens to enable remodeling [38]. For example, stromelysin, a type of matrix metalloproteinase, can remove type IX collagen molecules from the surface of type II fibrils so that the fibrils can add additional type II molecules to increase diameter [44,45]. This method of catabolism allows chondrocytes to remodel components of the matrix incrementally without removal of the entire architecture. Chondrocytes lay down matrix through anabolic effects stimulated by growth factors such as platelet-derived growth factor, insulin like growth factor, fibroblast growth factor and transforming growth factor- β . Additionally, inhibitors of catabolism including tissue inhibitor of metalloproteinase, plasminogen activator inhibitor and kallikrein counterbalance matrix degradation [46]. In OA numerous etiologies such as trauma or aging disrupts chondrocyte metabolism to favor catabolic processes causing net degradation of the cartilage matrix [47].

Collagen accounts for approximately 50% of the dry weight of the cartilage matrix [32] and can be thought of as the endoskeleton of the articular cartilage, providing structure, compressive resistance and tensile strength [38]. Type II collagen makes up 80-90% of the collagen and is a fibril-forming collagen along with smaller amounts of type I, III, V and XI collagen [44]. Non-fibril forming collagens include type IX that comprises about 1% of cartilage collagen and is covalently linked to type II collagen and thought to mediate fibril-fibril, fibril-proteoglycan interactions [48-50]. Like chondrocytes, collagen also has a highly anisotropic structure that becomes organized during growth and gains its mature structure by puberty [35]. Collagen is differentially organized from the superficial through the deep zones, and also depending on proximity to the chondrocyte [35]. A study of porcine cartilage found that in young animals the collagen fibers were parallel with the cartilage surface, with lower collagen content overall. As the animals reached maturity the collagen fibrils in the superficial zone remained parallel with the surface, but deeper collagen fibrils altered orientation to be perpendicular with the cartilage surface, forming the typical arcade structure described by Benninghoff [51]. The orientation of the parallel collagen fibers with small diameter close to the joint surface with deeper collagen fibers orientated in a perpendicular and arcade fashion was first described in 1925 [52]. The

findings continue to provide structural explanation to the ability of articular cartilage to resist compression and shear forces on its surface.

Proteoglycans make up approximately 35% of the dry weight of cartilage matrix [32]. There are different types of proteoglycans, the most common of which is aggrecan (approximately 85% of the proteoglycans). All proteoglycans have a central protein core that is attached to multiple glycosaminoglycans via link proteins, with a resulting structure that resembles a bottlebrush. Glycosaminoglycans are negatively charged salts, such as chondroitin sulfate and keratan sulfate, they are responsible for maintaining the compressive properties of articular cartilage because by steric hindrance between similarly charged glycosaminoglycans and a high binding affinity for cations like Na^+ and therefore water molecules [53]. High water binding efficiency of proteoglycans causes cartilage to be 70-80% water [32]. As chondrocytes mature they are able to produce a larger amount of proteoglycans with higher glycosaminoglycan content, however as chondrocytes age quality again reduces by production of shorter proteoglycan chains [54]. Hyaluronic acid is produced by chondrocytes mainly in the transitional zone; it binds to collagen and aids the retention of proteoglycans [38].

Early physicians and scientists believed that articular cartilage was completely incapable of repair [55,56], partly due to lack of neural innervation and vasculature [57,58]. This theory was revised when chondrocytes were capable of division when isolated from cartilage matrix [59,60], leading to understanding of potential mechanisms of cartilage repair: 1) intrinsic repair from short-lived increase in chondrocyte metabolism and limited ability to divide, 2) extrinsic repair from chondrocytes or stem cells in other joint tissues, such as subchondral bone [60,61], and 3) matrix flow where edges grow toward the center by forming wave-like flaps [62,63]. It is now known that the ability of cartilage to undergo intrinsic repair is poor rather than incapable. Small partial thickness defects do not heal spontaneously, however defects that are $\leq 5\text{mm}$ and full thickness (grade IV, superficial osteochondral, IRCS) will undergo repair [64-67]. The reason for this is that chondrocytes migrate slowly at $5\text{-}10\mu\text{m/h}$ in the ECM [68,69] compared to 20 to $720\mu\text{m/h}$ for human smooth muscle [70] and neutrophils [71], respectively. In comparison, full thickness defects allow progenitor stem cells and growth factors to be introduced from bleeding of the underlying subchondral bone [72]. Full penetration of the subchondral bone layer via microfracture further enhances repair [73]. However, cartilage defects heal with fibrocartilage

that is essentially scar tissue with a lower resistance to mechanical stress [74]. Furthermore, the process of cartilage injury is an inciting factor of OA [75-78]; subsequent degradation during OA occurs because of the limited ability of cartilage to repair and ongoing tissue degradation.

Ligaments

Diarthrodial joints are maintained in anatomic alignment during their normal range of motion by ligaments that can either be extra or intra-articular. Ligaments stabilize diarthrodial joints in response to tensile load and originate and insert on different bones at junctions called entheses or osteoligamentous junctions. The ligament structure allows the transfer of forces to and from the skeleton. Although ligaments appear as single structures, their multiple fascicles allow segmental loading at osteoligamentous junctions. Ligaments are primarily composed of type I collagen (85%) as well as types III, VI, V, XI, and XIV collagen, proteoglycans, elastin and glycoproteins [79]. Collagen molecules form a triple helical structure that makes up crimped fibrils, which make up fibers that are cross-linked to increase strength [79]. When load (tension) is applied to ligaments, the crimp initially straightens and the tendon will return to its original length when load is removed. If load is increased beyond when all collagen fibers are straight, they will continue to absorb load until their failure point is reached and tearing occurs as in acute ligament injury (Fig. 1.1) [79]. In cases of repeated stress injury, stretch of ligament for a prolonged period prevents restoration of original crimp pattern and the resulting laxity leads to joint instability and eventual OA [80]. The two processes that lead to this are creep, where constant load over time causes ligament elongation; and load relaxation, where constant elongation causes reduced strain in the ligament over time.

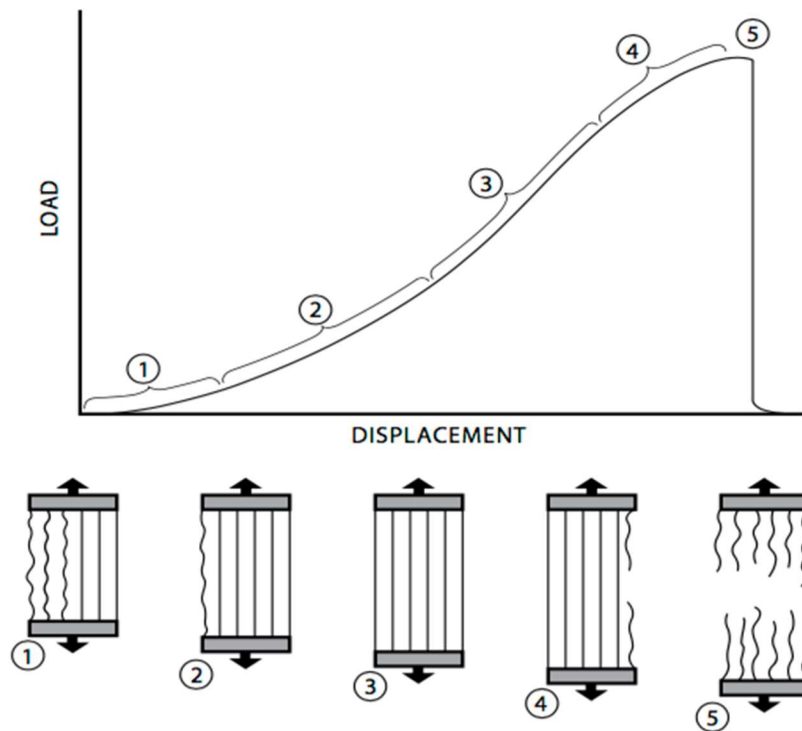


Figure 1.1 – Ligament loading

As load increases more ligament fibers straighten, allowing increased length without structural damage (1,2) the collagen fibers continue to absorb load until failure (4, 5) [80]ⁱ

The link between ligament injury and subsequent development of OA is seen in both naturally occurring and experimentally induced OA. A systematic review of 7 prospective and 24 retrospective studies on OA following anterior cruciate ligament injury found that joint laxity was a commonly reported risk factor for development of OA [81]. Eighty-two percent of female [82] and 78% of male [83] soccer player knees with anterior cruciate ligament rupture developed radiographic signs of OA. Cruciate ligament transection has been utilized to induce OA in multiple species including mice [84], rabbits [85], dogs [86] and sheep [87]. Instability resulting from ligament damage causes a physical crushing of articular cartilage as bone ends articulate in an abnormal manner. Subchondral bone also remodels in accordance to Wolf's law due to increased load in abnormal locations within the joint. However, instability does not account for all the pathophysiological alterations occurring after ligament injury. Thirty percent of patients that undergo surgical fixation of cranial cruciate injury subsequently develop PT-OA that is

ⁱ Reprinted from Journal of Prolotherapy, 3/4, Hauser RA and EE Dolan, Ligament injury and healing: an overview of current clinical, 836-846, Copyright (2011), with permission from Hauser RA, MD and the Journal of Prolotherapy.

indistinguishable from primary OA [7,23], and surgery was not found to benefit the outcome of female or male soccer players with cranial cruciate ligament rupture [82,83]. Reasons for this phenomenon are discussed in detail in the section on OA initiation.

Subchondral bone

The mineralized tissues of the epiphysis consist of articular calcified cartilage, SCB and trabecular bone. SCB is the dense layer of bone that underlies articular calcified cartilage and is composed of the SCB plate and subchondral trabecular bone (Fig. 1.2). The purpose of SCB is to provide the articular surface with support and shock absorption. The trabeculae of the trabecular portion of SCB are oriented so as to absorb and disperse loading from the joint surface to the trabeculae of epiphyseal and metaphyseal trabecular bone that further disperse load before it reaches the cortical bone of the diaphysis. Exercise has been found to have little effect on the structural or mechanical properties of articular calcified cartilage [88,89]. However, equine SCB, which is composed of cortical bone, responds to load by thickening and increasing bone mineral density [90-94]. Trabecular bone of equine metacarpal/metatarsal epiphysis responds to exercise by increasing the number of trabeculae as well as their thickness, by depositing new bone around existing trabeculae, in what appears microscopically to be a rapid response [95,96].

Alterations in SCB density and thickness is related to the peak strains encountered during exercise [91,94,97]. There was no difference in bone mineral density response in young horses given long term light conditioning exercise compared to controls at pasture [92,98], but there was a significant response in horses that were given race training or treadmill exercise compared to controls [91,97,99]. Similarly, the spatial distribution of high bone density relates directly to the load path created during exercise by opposing bones, such as distinctive pattern of bone mineral density change due to contact of the proximal sesamoid bones on the distal metacarpal epiphysis in horses [93,97].

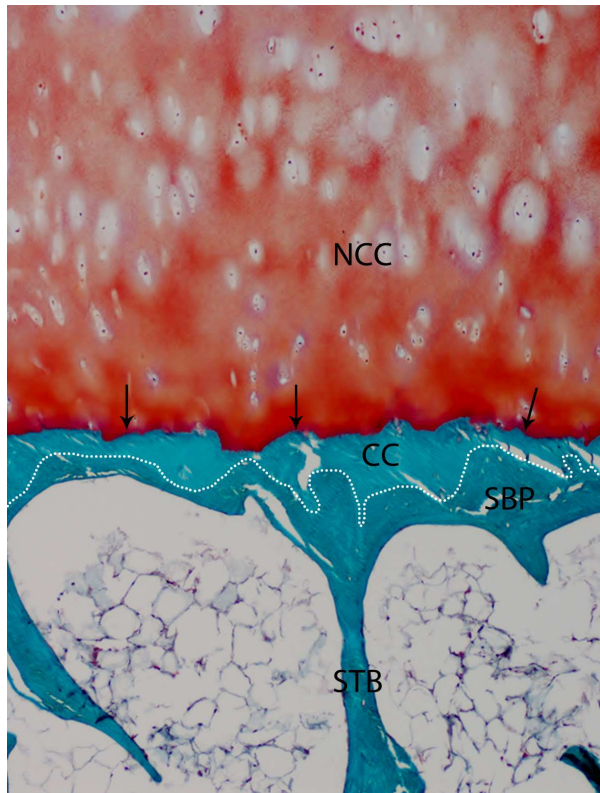


Figure 1.2 – Subchondral bone histopathology

The relationship between non-calcified articular cartilage (NCC), calcified cartilage (CC), subchondral bone plate (SBP) and subchondral trabecular bone (STB). Tidemark depicted by arrows and dotted line is the junction between CC and SBP. [100]ⁱⁱ

An abnormal response of SCB or a normal response to over loading can be found in both humans and horses affected by osteoarthritis. Early in the investigation of human osteoarthritis, trabecular thickening and clustering of the subchondral trabecular bone resulted in bone mineral density increase of the SCB [101]. Additionally, a higher percentage of very high bone mineral density in the palmar/plantar regions of the metacarpal/metatarsal condyles in horses with osteoarthritis has been observed [102]. Abnormal joint biomechanics, such as increased loading, have been found to reactivate ossification centres causing bone thickening and subsequent thinning of articular cartilage through its replacement with bone [103]. Microcracking, microfracture [104] and evidence of osteoclastic remodeling [105] have been found to occur within areas of very dense bone in the palmar region of the equine metacarpal condyles and in thickened SCB of human osteoarthritis patients [103]. Microcracks and areas of osteoclastic

ⁱⁱ Reprinted from Arthritis research & therapy 15/6, Li G, J Yin, J Gao, TS Cheng, NJ Pavlos, C Zhang and MH Zheng, Subchondral bone in osteoarthritis: insight into risk factors and microstructural changes, 233-235, Copyright (2013), with permission from BioMed Central.

remodelling combine to form lytic lesions that are found within palmar regions of thickened bone in horses with osteoarthritis [102].

SCB damage has both detrimental structural and physiological effects on the overlying articular cartilage. Physical SCB changes that affect articular cartilage range from SCB thickening to microfracture. As the SCB remodels in response to increased or abnormal load in the pathogenesis of osteoarthritis, there is a thinning of the articular cartilage. This change is seen evident as advancement of the tidemark (junction between articular cartilage and calcified tissues), and is coupled with histologic duplication of the tidemark, increased mineralization around vascular channels, calcified cartilage thickening and increased vascular invasion [106]. Furthermore, microfracture that occurs in areas of thickened SCB weakens it, causing concavity at the SCB surface and direct damage to the overlying articular cartilage. For horses, this is commonly seen in a condition referred to as palmar/plantar osteochondral disease or traumatic overload arthrosis [107].

Alterations in SCB are mediated through the actions of osteoclasts that resorb bone and osteoblasts that lay down new bone. OA affected joints have osteoblasts with altered phenotype and gene expression, for example they have elevated alkaline phosphatase and osteocalcin levels, key mediators of bone absorption and deposition, respectively [108], and also can have altered levels of the growth factor TGF β and cytokines IL-6 and PGE₂ [109]. There is mounting evidence of cross talk between osteoblasts and chondrocytes [110]. An *in vivo* model that separated chondrocytes from sclerotic bone derived or normal bone derived osteoblasts found that chondrocytes in the presence of sclerotic osteoblasts had increased production of matrix metalloproteinases but decreased aggrecan production [111]. Such cross talk supports findings of articular cartilage protection when treatments that prevent subchondral bone remodeling such as bisphosphates [112], calcitonin [113,114] and osteoprotegerin [115] are used experimentally.

Synovium and joint capsule

Along with the intra and peri-articular ligaments, the joint capsule helps to keep the diarthrodial joint stabilized. The joint capsule is fibrous and is contiguous with the periosteum [116]. The synovial membrane lines the internal surface of the joint capsule and is divided into two layers. The subintimal layer is composed of fibrous and vascularized areolar tissue, and the intimal layer

directly communicates with the joint cavity and determines the content of the synovial fluid due to secretory and phagocytic functions. The intimal layer is 1-3 cell layers thick and the cells are called synoviocytes. There are 3 different types of synoviocytes based on immunohistochemistry: fibroblasts, macrophages and dendritic cells [117]. Based on electron microscopic phenotypes, synoviocytes are classified into two different types (Type A and B) [118]. The macrophage-like synoviocytes are referred to as type A synoviocytes, and act to remove unwanted waste or cartilage breakdown products from the synovial fluid (phago-/pinocytosis) [119]. Type B synoviocytes are fibroblast-like and produce hyaluronan, lubricin, collagens and fibronectin for the synovial fluid [119,120]. Together, the products of type B synoviocytes ensure free movement of the synovial joint through lubrication. Lubricin is a superficial zone protein, binding to the surface of both synovium and cartilage acting as the main “lubricant” [121]. Hyaluronan determines synovial fluid viscosity and volume especially during exercise, which may have a protective “cushioning” function for adjacent joint tissues [122].

The structure, cellular composition and function of the synovial membrane make it a key tissue for the initiation, propagation and clinical signs of OA. The intimal layer does not have a basement membrane, which allows a high degree of filtration between the synovial fluid and peripheral circulation. During inflammation of the synovium (synovitis) joint effusion occurs by exudate accumulation i.e. overspill of synovial tissue edema created by increased vascular permeability [123]. The intimal layer has direct communication with the underlying subintimal layer, which contains vascular and lymphatic capillaries as well as innervation to aid in proprioception and perception of pain during joint swelling and synovial inflammation [116,123]. The close proximity of the intimal and subintimal layers with the peripheral circulation allows infiltration of inflammatory cells following joint trauma [124], and during the course of OA [125]. In normal synovium inflammatory cells are rare to absent and immunohistochemistry shows no staining for the inflammatory cytokines $\text{TNF}\alpha$ or $\text{IL-1}\beta$ [123]; however, during OA the synovium becomes the main site for production of inflammatory cytokines and propagation of inflammation [126].

Etiology of OA: Inflammation unites multiple inciting factors

An OARSI-FDA initiative in 2011 is the most recent attempt to define the disease state of OA and recognizes the complexity and variability of OA etiology. It defines OA as a representation of failed repair of joint damage that could have occurred due to stress of any of the joint tissues discussed in the previous section[7]. Such stresses may result from biomechanical [127], biochemical [128] and/or genetic factors [129]. Meta-analysis of case-control or cohort studies have found that main risk factors for the development of knee OA in humans are age, previous joint injury (pooled OR 3.86, 95% CI 2.61-5.70) and obesity (pooled OR 2.63, 95% CI 2.28-3.05) [130]. There are no similar case-control or cohort studies for horses; however, joint injury, age, over-use, conformational defects and developmental orthopedic disease have all been implicated as risk factors [30]. Central to ageing, obesity and joint trauma are inflammatory processes and the contribution of low grade inflammation in the initiation and propagation of OA regardless of etiology is now widely accepted [7,131].

As cells age they undergo nine detrimental alterations including telomere attrition, genomic instability, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, stem cell exhaustion, altered intercellular communication, mitochondrial senescence and cellular senescence [132]. The term “inflammaging” has been proposed for the combination of these processes due to inflammatory mediators released by aging cells [133], as well as increased levels of systemic inflammatory markers including IL-6, C-reactive protein and TNF α activity but a decline in anti-inflammatory markers found in aging people [134,135].

There are two main mechanisms proposed for the increase in intra-articular inflammatory cytokines with age: advanced glycation end products (AGEs) and chondrocyte senescence. AGEs increase naturally in aging cartilage cause increased cross-linking of collagen molecules resulting in increased susceptibility of cartilage to fatigue failure [136,137]. They also bind to cell surface receptors that are increased in OA such as the Receptor for Advanced Glycation End-products (RAGE) [138]. The RAGE has been found to stimulate signaling pathways involved in catabolic processes and chondrocyte senescence, such as MMP expression [139,140] and chondrocyte hypertrophy [141].

Another mechanism proposed for increased inflammatory cytokines in aging cartilage is chondrocyte senescence [131,142,143]. Cellular senescence was originally described as the exhaustion of cell division (replicative senescence) [144], however cells that have low replicative capabilities undergo another type of senescence in which cells produce high levels of inflammatory mediators termed the senescence-associated secretory phenotype (SASP) [145]. Mature chondrocytes have poor replicative capabilities and the increase of IL-1 β induced MMP-13 production with age suggests that chondrocytes gain SASP [146]. SASP may also be a mechanism involved in OA development with over-use or repetitive training as low-grade repetitive loading to the articular cartilage has been found to induce chondrocyte senescence [147].

Obesity is of increasing concern for modern society, and the likelihood of acquiring OA increases with increasing BMI in a dose-response manner [148]. The effect of obesity on equine OA has not been investigated; however, prevalence of equine obesity (BCS $\geq$ 7-8/9) is 18.7-35% depending on study location [149,150]. Obesity was originally thought to have a solely biomechanical influence on initiating OA [151]. Cartilage responds to loading by altering chondrocyte metabolism but the direction and degree of response is dependent on static or dynamic loading characteristics. Under static compressive load chondrocytes suppress aggrecan and collagen production, but dynamic conditions with a frequency above 0.01-0.1Hz threshold increases production of matrix components [152]. However, cyclic overloading that occurs with obesity and forced or high-intensity exercise promotes cartilage catabolism and OA [153]. The biomechanical theory of obesity related OA recently came into question with strong evidence that the risk of hand OA in obese people is doubled [154]. An etiology of obesity related OA that recognizes inflammation is now accepted because the biomechanical load theory cannot explain the increased risk in non-weight bearing joints and there is increased understanding of the endocrine nature of adipose tissue [155,156].

Metabolism of articular cartilage is mediated via inflammatory cytokines, particularly IL-1 β [38,157]. Adipose tissue produces cytokines and adipokines that have structures resembling pro-inflammatory cytokines. The main inflammatory cytokines produced by adipose tissue and adipocytes in particular, are IL-1 β , TNF α [158-160] and IL-6 [161,162]. Although adiposity and fatty acid metabolism are often linked, it has been found that non-obese rats fed a high fat diet

have increased levels of inflammatory cytokines in their adipose tissue [163]. High fat diets cause decreased glycosaminoglycan content and aggrecan expression in rabbits regardless of body weight [164], and have increased cartilage degeneration in a murine tibial plateau fracture model of post traumatic OA compared to those fed a normal diet [165]. Obese people have increased systemic markers of inflammation associated with adipose tissue deposits including IL-6, macrophage chemoattractant protein-1 and TNF α [166]. The link between adiposity related systemic inflammation and OA was clearly demonstrated in mice fed a normal or very high fat diet that developed OA in parallel with systemic inflammation and were protected by having moderate exercise [167]. Together the data from obesity related OA studies demonstrates that inflammation caused by obesity is critical for initiating OA [155].

A history of joint trauma is consistently found to increase the likelihood of OA and as a result was the highest risk factor in a meta-analysis of 85 epidemiological studies [130]. In another recent meta-analysis that followed patients with anterior cruciate ligament injury for an average of 14 years, there was no difference in eventual development of radiographic signs of OA with or without reconstructive surgery despite increased stability in the operated group [168]. For all forms of joint trauma, clinical OA developed in over 30% of patients despite surgical management to promote long term stability and joint congruity [7,23].

Initial inflammation following joint trauma contributes to the development of OA in both animal models and naturally occurring human disease [169-172]. The specific timing and relative amount of inflammatory cytokines have been demonstrated using a murine tibial plateau fracture model of PT-OA (Fig. 1.3) [173]. Following fracture, IL-1 β expression increased 720-fold within 4 hours and remained elevated greater than 200-fold to 3 days. The IL-1 β peak was the initial inflammatory mediator peak and was followed by an increase in TNF α that also peaked within the first 24 hours of injury. Following the peak of IL-1 β there was infiltration of lymphocytes into the synovium and an associated increase in chemokines. The inflammatory peak was short lived; within a week both IL-1 β and TNF α expression had decreased to pre-fracture levels. Inflammatory cytokine profiles of people with knee injury also show that IL-1 β , IL-8 and IL-6 peak within 24 [174] to 48 [175] hours of injury and reduced by 2 weeks after injury [175] with levels remaining mildly elevated compared to non-injured control joints [174].

An increase in biomarkers of cartilage breakdown occurred 24 hours after the inflammatory cytokine peaks [174,176].

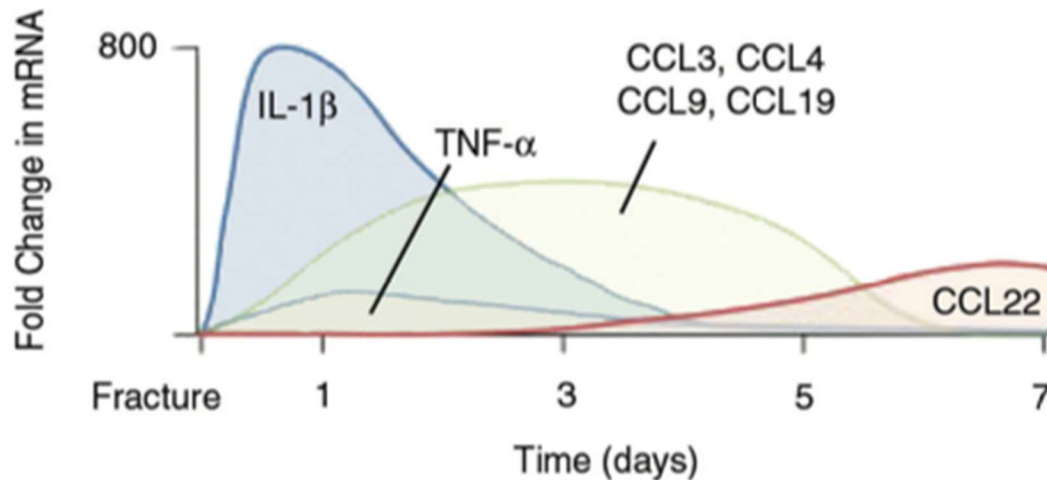


Figure 1.3 – Inflammatory events following joint injury

Temporal analysis of gene expression of inflammatory cytokines and chemokines following closed intra-articular tibial plateau fracture in mice [173]ⁱⁱⁱ

Inflammation following joint trauma reflects responses from all joint tissues. The degree of synovitis and levels of inflammatory mediators are correlated with injury severity, for example the degree of fracture comminution [177]. Following closed intra-articular fracture synoviocytes had the earliest gene expression of IL-1 β and TNF α compared to chondrocytes [173] and increased levels of inflammatory cytokines and cartilage degradation products were proportional to the degree of synovitis rather than viable chondrocytes [177]. Initial production of cytokines by synoviocytes promotes infiltration of the synovium with mononuclear cells that continue cytokine production and are responsible for a subsequent peak in chemokines [173,178]. Following intra-articular fracture there is significant loss of chondrocyte viability [77,177] with histologic and biomarker changes consistent with OA [76]. Direct application of extra-physiological point load to the medial femoral condyle of horses caused mild lameness, increased cartilage degradation products and histologic changes consistent with OA by 84 days post injury [179]. Amelioration of local inflammation may be critical to chondrocyte viability

ⁱⁱⁱ Reprinted from Arthritis Rheum, 65/3, Lewis JS, Jr., BD Furman, E Zeitler, JL Huebner, VB Kraus, F Guilak and SA Olson, Genetic and cellular evidence of decreased inflammation associated with reduced incidence of posttraumatic arthritis in MRL/MpJ mice, 660-70, Copyright (2013), with permission from John Wiley and Sons.

following trauma, as nitric oxide inhibitor prevented chondrocyte death after impact injury of canine articular cartilage [78]. However, direct injury to articular cartilage does not produce high levels of inflammatory cytokines or synovitis, as seen in clinical or experimental models of PT-OA [179]. Joint trauma that preferentially damages the SCB also results in OA and eventual articular cartilage degeneration [180]. Importance of SCB damage to the degree of inflammation is seen by the higher levels of inflammatory cytokines produced when there is osteochondral involvement [176]. There is evidence that other damaged joint tissue, such as the meniscus are additional sources of inflammatory cytokines after joint injury [181,182].

Reduction of inflammation within the first 48 hours of joint injury can ameliorate the progression of OA. A model of PT-OA without instability, created by drilling into the subchondral bone of the femoral notch, had decreased markers of inflammation and cartilage degeneration when treated with dexamethasone [183]. Reduced markers of cartilage degeneration, decreased osteophytes and bone sclerosis, and decreased lymphocyte infiltration of the synovium have been observed using a single dose of recombinant IL-1ra immediately following trauma [184]; however, no benefit was gained by repeated administration [185]. Although the robust inflammatory peak decreases after injury, low levels of inflammation are sustained [175,186,187] that eventually result in the classic OA phenotype [172]. Although amelioration of OA has been seen by anti-inflammatory treatment within 24 hours of joint trauma, it is still unknown if chronic perpetuating inflammation can be resolved [188].

It is critical to note that the initiation of OA is multifactorial [7] and only three key etiologies have been discussed. The importance of inflammation to the initiation of OA is clearly demonstrated, albeit different in regard to source, cause or degree. Reduction of the inflammatory mediators that can initiate the OA process is a topic of intense research and it is likely that different approaches will need to be taken depending on exact inflammatory source or cause. Despite different initiating causes, OA progresses in a similar manner to produce tissue wide degeneration.

Progression of OA: A cycle of inflammation and tissue degradation

OA was originally considered a cartilage-driven disease because chondrocytes have low metabolic activity with little to no ability to repair articular cartilage damage [37]. OA was

termed “osteoarthritis” because it was thought to be a non-inflammatory disease, as opposed to Rheumatoid Arthritis (RA) with profound inflammation [9,189]. Although the degree of synovial mononuclear cell infiltration and pro-inflammatory mediators in OA are lower than RA [190,191], the central role of inflammation to the initiation and propagation of OA is now well accepted [7,142,172,189]. This has been partly due to the progress of molecular biology to detect low levels of inflammation in the cellular transcripts, but also because previously synovial tissue from OA patients was used as a “non-inflamed” control for RA synovial tissue [9], which gave a false representation of the inflammatory state of OA affected joints. Although the relative importance of inflammatory mediators from different joint tissues is complex, they are produced by cartilage, bone and synovium, making all tissues important to the progression of disease [143,192,193].

The key inflammatory cytokines involved in OA are IL-1 β and TNF α , which are both produced by chondrocytes, synoviocytes, infiltrating mononuclear cells and osteoblasts [193]. IL-1 β produces increased cartilage damage compared to TNF α [194,195]; however, when both are injected into rabbit knees cartilage destruction is increased compared to each used alone [196,197]. IL-1 β and TNF α have multi-faceted effects: they stimulate chondrocytes to produce cartilage degrading proteinases, contribute to chondrocyte apoptosis and hypertrophy, cause monocytes to infiltrate the synovium and stimulate bone remodeling of subchondral bone. IL-1 β and TNF α continue to be produced at low, chronic levels through stimulation of the innate immune system by ECM breakdown components [178].

IL-1 β and TNF α cause down-stream production of other inflammatory cytokines and chemokines including IL-8 [198], IL-6 [199], monocyte chemoattractant protein 1 [200], NO and PGE₂ [193]. Some pro-inflammatory cytokines, such as IL-6 and PGE₂ may act to regulate inflammation as well as propagate it. For example IL-6 in combination with IL-1 β up-regulates MMP-1 and MMP-13 in human and bovine chondrocytes [201,202]; however, IL-6 can also induce IL-1 α expression of monocytes [203], IL-6 deficient mice have accelerated subchondral bone sclerosis [204], and intra-articular IL-6 injection is chondroprotective in IL-6 deficient mice with collagenase induced arthritis [205]. PGE₂ is associated with inflammation because it reproduced fundamental signs of inflammation when injected into normal tissues and is present

in inflamed tissue [206]. However, PGE₂ has a complex role as an immunomodulator, which may be concentration and context dependent because only very low concentrations were anti-inflammatory in *in vitro* models of OA [207-209]. PGE₂ can regulate the inflammatory response of synoviocytes by regulating dimers of NFκB, inhibit MMP expression and apoptosis of chondrocytes, suppress cartilage collagen cleavage, and produce anti-inflammatory effects via transition of macrophages to an anti-inflammatory phenotype that produces IL-10 [4,208,210-214].

Progression in the synovium

Synovial inflammation (synovitis) is defined by production of inflammatory cytokines by synovial tissue and a mixed inflammatory infiltrate of activated B cells, T cells and macrophages [191,215]. Synovitis is involved in disease progression and has been recognized in both early (arthroscopic but no radiographic signs) and late stage (at arthroplasty) OA [215]. Studies investigating murine models of PT-OA have found predominantly activated macrophage infiltration as well as associated expression of inflammatory cytokines and chemokines following peak IL-1β expression by synoviocytes [173,177]. There is also prominent macrophage infiltration found in early human OA when arthroscopic but not radiographic signs of OA are present; and there is continued presence of macrophages through to end-stage OA [215].

Macrophage infiltration is a common and significant finding in OA. Recently SPECT-CT imaging found macrophage infiltration of OA synovium in 76% of knees with OA [216]. *In vitro* experiments have found that macrophages are key producers of IL-1β, TNFα, IL-6, IL-8 and MCP-1 in OA synovium and also produce MMPs 1 and 3 [217]. The production of MMPs may degrade adjacent areas of cartilage in proximity to synovitis [218]. In addition to cartilage damage, pain was reduced in murine collagenase arthritis by inhibition of macrophage-derived factors [219]. Given the potential for inflammatory mediator production and cartilage degradation, it is not surprising that the degree of synovitis in knee osteoarthritis is predictive of arthroscopic [218] and radiographic [220] progression of OA.

Synovitis not only affects the joint at the biochemical level, but also function of the whole joint. During OA or after joint injury, synoviocytes produce lower quantities of hyaluronic acid that

has decreased molecular weight [221-223]. Additionally, the concentration of lubricin is reduced, which not only affects boundary lubrication but also affects its ability as an articular surface barrier to pathologic molecules [224]. As inflammation and hyperplasia occur during synovitis, the permeability of the synovial membrane changes. Loss of hyaluronic acid from the joint space due to increased permeability is demonstrated by its ability to be used as a marker of OA [225] due to increased serum levels [226]. Synovitis has been linked with increased severity of pain and joint dysfunction. Synovitis was correlated with pain on joint palpation in people with hand OA [227] and has been directly associated with knee pain in numerous studies [228-231]. Synovitis not only increases the likelihood of experiencing knee pain almost 10-fold [228], but the degree of synovitis is directly proportional to patient's pain [230]. Likely as a result of increased pain, patients with synovitis also have reduced Lysholm scores indicating impaired knee function [229].

Progression in the articular cartilage

Regardless of the initiating factors of OA, the progression of cartilage damage follows a specific sequence of events: there is altered chondrocyte phenotype and metabolism, matrix degradation and osteophyte formation [232]. Chondrocytes drive matrix changes in response to increased matrix permeability, the presence of matrix breakdown products, alterations in cartilage mechanics and inflammatory cytokines.

Early in OA chondrocytes exhibit hyperactivity and anabolism that varies on chondrocyte location due to different exposure to inflammatory cytokines and the degree of compromise to the cartilage matrix [233]. In contrast to normal chondrocytes, that have low replicative ability, osteoarthritic chondrocytes proliferate to form clusters and/or become disorganized [234]. Cluster formation represents alteration of chondrocyte phenotype, regulated by the cytokine mediated transcription factor, NF- κ B [235], and has altered expression of growth factors and matrix components such as cartilage oligomeric matrix protein (COMP) [236]. Genes for other ECM components expressed by hyperactive chondrocytes include collagen type II and aggrecan [237]. This hyperactivity may represent early attempts of cartilage repair, however catabolic processes and chondrocytes with detrimental phenotypic changes overtake the disease process.

Hypertrophy and apoptosis represent alterations in chondrocyte phenotype that are detrimental to articular cartilage. Hypertrophy represents de-differentiation of chondrocyte phenotype that can act as a source of ECM degradation through the release of proteinases [238]. Hypertrophic chondrocytes are a source MMP 13 and type X collagen, but have reduced type II collagen production [239,240] and genes associated with de-differentiation are found in cartilage from early OA [241]. MMP 13 is particularly important to the pathogenesis of OA as it is the main proteinase that cleaves type II collagen [242]. Apoptosis affects 1-22% [243-245] of chondrocytes in cell clusters or individual cells at all stages of the disease process [234] and varies with location in the joint [245]. The key inflammatory mediators that induce chondrocyte apoptosis are NO [246], IL-1 β and TNF α [212], however the process is complex and influenced by the up-regulation of intra-cellular pathways, which may differ depending on stage and etiology of osteoarthritis [212,246]. Chondrocyte apoptosis and matrix degeneration are related; there is less proteoglycan staining in areas adjacent to apoptotic chondrocytes and the degree of matrix degradation on histology is related to the number of apoptotic cells in both people [243] and horses [247]. Reduction in viable chondrocytes reduces the ability for cartilage to self-repair; however, it is unknown if the process of chondrocyte apoptosis directly degrades ECM, or if the two occur in parallel in response to inflammatory mediators and reactive oxygen species [248].

With OA progression, anabolic processes of chondrocytes decline leaving an overwhelming imbalance toward catabolism [249]. Inflammatory cytokines, particularly IL-1 β and TNF α have been implicated in the imbalance between chondrocyte anabolism and catabolism *in vitro* and *in vivo* [157,250]. Catabolism of chondrocytes involves production of MMPs, ADAMTs (a disintegrin and metalloproteinase with thrombospondin motifs) and a reduced production of ECM components. MMPs and their closely related ADAMTs are protease enzymes that hydrolyze components of the ECM. There are 24 vertebrate MMPs that act on collagens, link proteins, aggrecan, and other protein components of the ECM such as fibronectin and decorin [251], while ADAMTs degrade proteoglycans. The finding that direct antagonism of IL-1 β and TNF α down-regulated expression of MMP-1, MMP-3 and MMP-13 from osteoarthritic chondrocytes and inhibited cleavage of type II collagen and breakdown of proteoglycans [250] highlights the importance of inflammatory mediators in the process of ECM degradation.

MMP-13 is important in the process of articular cartilage degradation because its main substrate is type II collagen [242], however all catabolic enzymes contribute to the breakdown of the ECM components, many of which can be detected in the blood stream as biomarkers [27,252,253]. The alteration of chondrocyte metabolism from an anabolic to catabolic state occurs in the superficial zones prior to occurring in the mid to deep zones [249]. Breakdown of type II collagen in the superficial layers means that proteoglycans have less “restriction” allowing them to gain more water content causing cartilage softening and swelling in the early stages of disease [254]. Surface fibrillation follows as the cartilage cannot withstand physiological stresses and begins to incur mechanical wear. As disease progresses chondrocytes of the deeper layers gain a catabolic state and cartilage wear exposes increasing amounts of the ECM to catabolic enzymes [234]. Although anti-inflammatory cytokines such as IL-10, IL-4 and IL-13 as well as tissue inhibitors of matrix metalloproteinases (TIMPs) are produced to counter-act catabolic processes, they are insufficient to dampen the cycle eventually resulting in complete destruction of the articular cartilage [156].

Activation of the innate immune system provides a critical pathway in the propagation of pro-inflammatory cytokines and chemokines in response to tissue destruction [255]. Breakdown products of the articular cartilage matrix can activate the innate immune system by direct activation as damage-associated molecular patterns (DAMPs) that are recognized by pathogen recognition receptors (PRRs). Both Toll-like receptors (TLRs) and RAGE are members of the PRR family, result in pro-inflammatory mediator production via NF κ B [255-257]. For example, fibromodulin can directly activate the complement cascade via the C1q receptor [258] and fragments from fibromodulin [138], hyaluronan [259] or collagen type II cause chondrocytes to up-regulate MMPs and pro-inflammatory cytokines via NF κ B signaling [260]. DAMPs also propagate synovial inflammation; analysis of human OA synovial fluid found proteins that acted via TLRs on synovial macrophages [261] and DAMPs initiated synovitis and disease progression in mice [262]. Different classes of PRRs may be involved in different tissue types, or in certain OA etiologies. For example, knock out mice with TLR2 deficiency decreased signs of collagenase induced OA but not in mice with destabilization of the medial meniscus [263]. What types of receptors are most critical in the progression of different OA etiologies is under investigation [178].

Progression in the subchondral bone

Radiological signs of OA including subchondral bone sclerosis and osteophytes have long been defining features of the disease [8]. Although subchondral bone sclerosis may only be observed in more advanced stages of the disease [264] and osteophytes are seen 4 weeks after anterior cruciate ligament transection in rabbits [265], alterations in the subchondral bone occur prior and affect other joint tissues [100]. Micro changes in the bone precede subchondral bone sclerosis as are evident in early OA and are associated with cartilage degeneration [266]. These micro changes represent early alterations in subchondral bone metabolism, seen by scintigraphic analysis [267]. Subchondral bone from OA patients has high levels of MMP-3, MMP-2 and the cytokines IL-1, TNF α and IL-8 [268]. As well as their own production of inflammatory cytokines and MMPs that propagate low-level inflammation and cause down-regulation of ECM production [111] and ECM degradation. Additionally, osteoblasts from OA bone stimulate chondrocytes to produce MMPs [111] including MMP-3 and MMP-13 [111] and express genes consistent with a hypertrophic phenotype [269]. Osteophytes represent periosteal lining growth-plate like formations that undergo hypertrophy and endochondral ossification [265] and may be influenced by similar mechanisms that cause subchondral bone remodeling in osteoarthritis [112]. MRI has allowed identification of bone marrow edema in sub-types of OA and associated it to severity of joint pain and cartilage lesions [270]. Bone marrow edema present at the time of hip replacement was associated with microfractures of the trabecular bone, areas of bone remodeling and significant cartilage loss [271]. Bone cysts represents the effects of physiological wear on weakened subchondral bone [100]. They are most commonly observed in end-stage disease and cause direct physical disruption to the overlying articular cartilage [272].

Summary

Through examples of age, obesity and trauma and description of pathological progression in key tissues of the joint, this review has demonstrated the importance of inflammation in all aspects of OA. The cycle of inflammation and tissue degradation continues to cause tissue degradation until end-stage disease, evident as eburnated articular cartilage, joint swelling, thickened synovium, subchondral bone cysts and osteophyte formation [234] (Fig. 1.4). An understanding of the cause and sequence of tissue degradation highlight the importance of early intervention as the

best way to prevent irreversible tissue damage [188]. However, patients with tissue damage detectable by MRI often show no clinical signs of disease [273]. Therefore, therapies that both stop the cycle of inflammation and tissue degradation, as well as repair already damaged tissues, is the herculean task of current OA research.

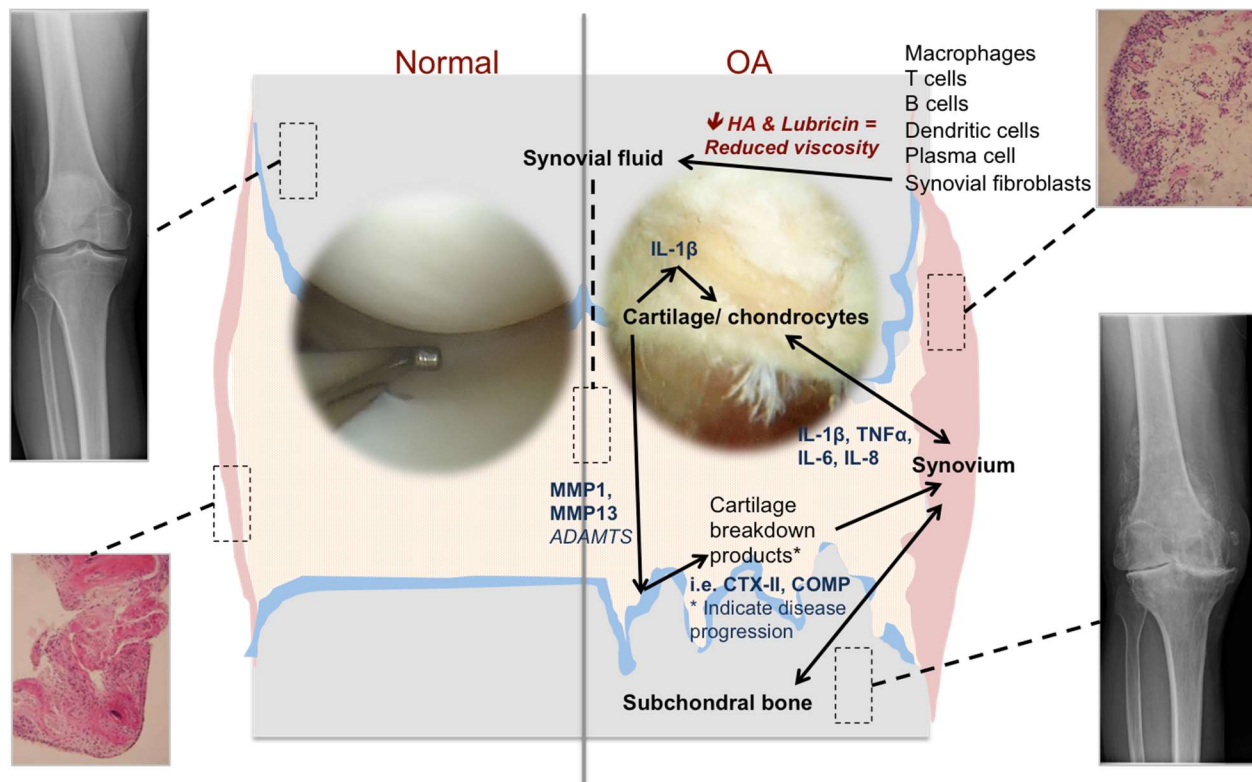


Figure 1.4 – Link between biochemical and gross pathogenesis of OA

The progression of OA is a cyclical process of inflammatory mediator production and tissue deterioration. Products of cartilage breakdown can contribute to further inflammation via the innate immune system and can be detected in the serum as biomarkers of disease. S. Bogers 2016.

Therapies for the treatment of OA

The ultimate goal of OA therapies is to halt disease progression and repair tissue damage while reducing clinical symptoms. Drugs that achieve altered disease progression due to amelioration or repair of damaged tissue are described as disease modifying osteoarthritic drugs (DMOADs). To date, sufficiently effective, recommended and FDA-approved DMOADs do not exist due to the multiple etiologies and pathologic pathways that act in the disease process, and conflicting results found in clinical trials [274,275]. Therefore, a multimodal approach with non-pharmacological therapies, non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular

corticosteroids, symptomatic slow acting drugs for osteoarthritis (SYSADOAs) and viscosupplementation is currently recommended for human OA [274,276]. Equine practice follows a similar approach [277]; however, is limited by plasma concentrations allowable during competition, and fewer available drugs for labeled use. A multimodal approach aims to relieve symptoms/clinical signs of OA and slow progression to end-stage OA. For humans, the end stage is joint replacement. In horses joint replacement is currently not feasible so arthrodesis is performed when possible, however many horses succumb to loss of use and wastage [26,278-280].

Anti-inflammatory pharmacological therapies

– NSAIDs

Anti-inflammatory therapies currently used for both human and equine OA are NSAIDs, intra-articular corticosteroids. NSAIDs act on the cyclooxygenase pathway to inhibit the production of prostaglandins from arachidonic acid. As there are two cyclooxygenase isoforms, COX-1 and COX-2, that are responsible for cytoprotective prostaglandins and inflammatory prostaglandins, respectively, both unspecific and COX-2 specific (COXIBS) NSAIDs are available for the treatment of human [281-283] and equine [284,285] osteoarthritis. COXIBS were developed to prevent gastro-intestinal, renal and cardiovascular side effects related to COX-1 inhibition, however, some continue to have side-effects [283]. Topical NSAIDs, such as diclofenac, have increased safety profile as their blood level after absorption reaches 0.4-2.2% of the maximum blood concentration reached with oral diclofenac in humans [286]. NSAIDs provide fast pain relief from OA symptoms but are unable to alter the progression of disease [276].

NSAIDs have been recommended unanimously for symptomatic human OA by meta-analyses and systematic reviews with or without topical NSAID (i.e. diclofenac) [274,276,287]. Evidence for the use of NSAIDs in equine OA stems mainly from *in vivo* studies that investigate alleviation of lameness [284,288,289], and *in vitro* studies that investigate effects on cartilage [290-293]. Oral phenylbutazone is a commonly used, cost-effective NSAID that reduced lameness in experimental synovitis [288], but its effects on matrix synthesis are controversial due to decreased synthesis of proteoglycan [290] and collagen type II [294]; however, other studies have found no effect on cartilage metabolism [295,296]. Flunixin meglumine is another

commonly used non-specific COX inhibitor, which reduced lameness in 74% of horses with mixed musculoskeletal disorders [297]. However, significant limitations for oral non-selective COX inhibitors are potential for renal and gastro-intestinal toxicity when used chronically [298] and that the United States Equestrian Federation only allows firocoxib or topical diclofenac on an on-going basis [299].

Topical diclofenac and firocoxib, a selective COX-2 inhibitor, have a potentially improved safety profile for long-term use [300] and have less detrimental effects on articular cartilage [301]. Used separately, both were effective at alleviating pain associated in an experimental osteochondral fragment model [301], and in naturally occurring osteoarthritis [284], respectively. Topical diclofenac improved proteoglycan content of cartilage compared to non-treated controls and had less radial carpal bone sclerosis and gross cartilage erosion than phenylbutazone treated horses despite being an equivalent efficacy for lameness [301]. Firocoxib has been found to reduce lameness in 79% [302] to 85% of horses with naturally occurring osteoarthritis, with no difference in efficacy compared to phenylbutazone [284]. The use of these NSAIDs is still limited in racehorses under the jurisdiction of the RMTC [303], and the FEI [304], that governs international level equestrian sports.

– Corticosteroids

Corticosteroids bind to nuclear glucocorticoid receptors and act via inhibition of NF κ B to reduce accumulation of inflammatory cells, production of neutrophil superoxide, MMPs and inflammatory cytokines via prostaglandin and leukotrienes [305,306]. Intra-articular administration of corticosteroid is recommended for moderate to severe OA in humans, but is inappropriate for OA in multiple joints, or early OA due to increased risk of multiple intra-articular injections and the availability of other effective symptom relieving drugs like NSAIDs [274-276]. Although meta-analyses and systematic reviews have found that corticosteroids are effective in reducing pain and enhancing joint function in people, the effect is relatively short-lived: pain relief and functional improvement are approximately 4 weeks with no significant difference between triamcinolone acetonide (TA), methylprednisolone acetate (MPA) and betamethasone [307-309].

A variety of intra-articular corticosteroids are used for the treatment of equine OA including MPA, TA, betamethasone and isoflupredone acetate [310], but only MPA and TA have been evaluated both *in vitro* and *in vivo*. MPA has the longest systemic half-life of 19.2 hours [311] and detection time of 77 days in the synovial fluid [312]. Although MPA (100mg/joint) decreased PGE₂, synovial hyperplasia and vascularization in a carpal osteochondral chip model of osteoarthritis, there was evidence of increased articular cartilage damage [313]. The same dose of MPA used in normal carpal joints injected every 14 days during treadmill exercise showed a detrimental reduction in the compressive stiffness, thickness and shear modulus of the cartilage [314]. *In vitro* studies suggest that the detrimental effects were mainly due to proteoglycan depletion in normal cartilage [315,316]. However, these detrimental effects on cartilage may be related to the presence of inflammation and the dose used. For example, when used in an LPS model of synovitis 100mg did not cause reduction in proteoglycan synthesis [317] and IL-1 stimulated cartilage explants treated with and 0.5mg/mL MPA had reduced proteoglycan loss compared to IL-1 only controls [318]. Doses of MPA >0.01mg/mL reduced proteoglycan loss in normal cartilage but 1mg/mL was needed to prevent proteoglycan loss in response monocyte conditioned medium [319]. As a result of these findings, many practitioners do not use methylprednisolone in high motion joints and choose to use a lower dose [310]. Even so, *in vivo* dose-response studies are lacking and positive treatment responses were observed at supraphysiologic levels of inflammatory stimulation.

TA is the second most common intra-articular corticosteroid (26%) after MPA (45%) used by members of the American College of Rheumatology in people [320]. Veterinarians most commonly use TA for high motion joints (77%) and reserve MPA for low motion joints (72%) in horses [310], likely due to evidence of detrimental effects of MPA in exercising horses [313,316]. MPA is favored over TA for low motion joints due to its perceived length of activity [310,312], although no differences have been demonstrated between TA and MPA for the degree or length of amelioration of lameness in distal tarsal joints [321]. Like MPA, intra-articular TA reduces lameness and signs of synovial inflammation associated with an experimental osteochondral fragment model of OA, but the gross deterioration of articular cartilage was not observed [322]. However, down-regulation of proteoglycan and collagen synthesis was evident in healthy cartilage *in vivo* [323,324]. In inflamed cartilage there are confounding results as to the chondroprotective effects of TA, with protection of chondrocyte morphology from the effects

of LPS [325] and reduced proteoglycan metabolism of IL-1 stimulated chondrocytes [326] found in two studies, but another study found that proteoglycan synthesis was reduced in IL-1 stimulated cartilage explants [315].

Betamethasone is used in high motion joints by 19% of veterinarians and by 7% in low motion joints of horses [310]; however there is less evidence of its effect on lameness or cartilage metabolism than MPA or TA. A study that used betamethasone in a bilateral carpal osteochondral fragment model of OA, failed to show differences in lameness or cartilage histology with treatment [327]. There are no peer-reviewed studies on the effects of cartilage metabolism; however, similar levels of chondrotoxicity were observed between MPA, TA and betamethasone in a recent dissertation [328]. Aside from the pharmacokinetic data to calculate drug withdrawal times [329], little to no information is available for the intra-articular use of isoflupredone acetate in horses even though it is used by 9% of veterinarians in high motion joints and 7% of veterinarians in low motion joints [310].

Given the degree of symptomatic improvement given by corticosteroids, and their widespread use in the treatment of equine OA, adjunct therapies have been added in an attempt to ameliorate the detrimental effects on articular cartilage. *In vitro* studies have found that glucosamine HCL [330] and sodium hyaluronate [318] protect cartilage explants against the proteoglycan losing effects of MPA. Hyaluronate added to triamcinolone was no different to triamcinolone alone in protecting against proteoglycan loss from chondrocyte pellets, but addition of hyaluronate did increase proteoglycan synthesis [326]. A recent randomized, blinded clinical trial of treatment with TA or TA with high molecular weight hyaluronate showed that the TA group had a higher success of lameness reduction 3 weeks after treatment (87.8%) vs. the combination (64.1%) [331] and contrasts against a similar human study [332]. It is still unknown if addition of a chondroprotective adjunct therapy, such as glucosamine, chondroitin or hyaluronate would improve outcomes of cartilage health *in vivo*, or result in long-term benefits over the use of a corticosteroid alone.

Disease Modifying Osteoarthritic Drugs (DMOADs)

– Chondroitin and glucosamine sulfate

Chondroitin sulfate (CS) and glucosamine sulfate (GS) are composed of a glycosaminoglycan unit component and are classified as SYSADOAs, which are drugs that are able to alleviate symptoms of OA but need to have a prolonged period of administration before the effects are observed [333]. The benefit of these drugs has become controversial because large randomized clinical trials have used different formulations, combinations, concentration and dosing of these drugs due to different international regulatory standards [274,333,334]. For example, until 2012 CS and GS were recommended via systematic analysis by the EULAR [335] and OARSI with a high level of evidence for symptom reduction and beneficial disease modifying effects (DMOAD) [287,336,337]. There was a synergistic effect to symptoms and disease when both CS and GS were administered [338,339]. However, when OARSI added updated meta-analyses and randomized controlled trials, the latest guidelines [274] found that glucosamine was uncertain for the amelioration of symptoms and not appropriate for disease modification. The reason for poor efficacy results is likely variation from studies using prescription vs. over-the-counter supplements, a large study [338] that used a glucosamine formulation with inefficient bio-availability (glucosamine hydrochloride) [340], and that discrepancies between short and long term disease modification were classified as DMOAD failure [341]. Given that when studies using prescription formulations of highly bio-available formulations (CS and GS) find clinically relevant pain and function effects, the recently published ESCEO recommendations consider SYSADOAs a safer and more holistic approach as the first step in knee OA management prior to other therapies with more potential for adverse effects [342].

There are many joint supplements for horses containing glucosamine and they are widely used. Glucosamine was the most common supplement given by horse owners in a study from New England [343], and the third most common for high-level event horses after electrolytes and plain salt [344]. However, there is large variability between the stated and actual content of glucosamine, ranging from 63.6% to 112.2%, and chondroitin sulfate, ranging from 22.5% to 155.7%, with no correlation between product price and compound purity [345]. Glucosamine products undergo degradation during their shelf life, resulting in 39.1% of equine products

containing less than manufacturer claims and 17.4% containing less than 30% of the claimed glucosamine [346]. The bio-availability and intra-articular concentration of glucosamine and chondroitin sulfate vary with formulation, route of administration and presence of inflammation in the joint. Like humans [338], glucosamine sulfate (GS) (20mg/kg [347]) was found to have increased bioavailability and intra-articular concentration after oral administration than glucosamine HCL (GH) and were sustained for 12 hours [347]. The intra-articular concentrations after oral administration for glucosamine are 0.09µg/mL (GH), 0.15µg/mL (GS) [347] compared to 1.49µg/mL (GH), 1.69µg/mL (GS) [347] when given intravenously. Although joint inflammation was found to increase intra-articular glucosamine hydrochloride concentrations approximately 4-fold [348] when LPS was used, this is likely a higher level of inflammation than commonly found in mild OA. Horses can absorb chondroitin sulfate (3g) given orally, with a trend toward better absorption of low-molecular weight products (8.0kDa) [349].

Glucosamine and/or chondroitin sulfate have been found to have chondroprotective effects on proteoglycan loss [350], MMP production [351-353], collagen II synthesis and histology of articular cartilage exposed to IL-1 or LPS. However, the concentrations used *in vitro* that have had these positive effects on cartilage health range from 10 [352]-1000µg/mL for GH [351] and 310-31,000µg/mL for GS [350]. Lower (0.1µg/mL) concentrations of GH decreased production of PGE₂ synthases but did not have an effect on proteoglycan or MMP production [354]. In the latter study, the GH concentrations used are approximately those seen *in vivo* with an oral dose; however, the other studies concentrations are 100-10,000 times higher [347,355]. Despite this discrepancy, *in vivo* studies that have tested a range of formulations of glucosamine and/or chondroitin sulfate have found improvements in lameness [356], stride length, range of motion [357] or a reduction in the need for intra-articular corticosteroid administration [358]; although, many lack appropriate control groups or randomization [359]. Overall, oral glucosamine and chondroitin sulfate have questionable status as DMOADs; however, their ability to improve clinical signs in some patients warrant them as a first line of treatment to potentially delay administration of NSAIDs or corticosteroids with increased detrimental side-effects.

Polysaccharide chains of amino sugar monomers (i.e. glucosamine, Pentosan) include polysulfated glycosaminoglycan/glycosaminoglycan polysulfate/PSGAG and pentosan

polysulfate. PSGAGs are not licensed for use in humans as adverse effects include bleeding diatheses, reversible hair loss and local hematomas, despite positive effects on OA symptoms [360]. Intra-articular PSGAG treatment of an osteochondral model of equine OA decreased sub-intimal fibrosis, vascularity and joint effusion, with a trend of decreased cartilage fibrillation [361]. However, an increase in septic arthritis after intra-articular administration was reported [362], likely due to potentiation of subclinical *Staphylococcus* contamination upon injection [363,364]. For this reason, most practitioners do not use PSGAG for intra-articular use [310]. Intra-muscular treatment with pentosan polysulfate in an osteochondral model of equine OA showed reduced cartilage fibrillation [365]. *In vivo* studies have not shown reduced lameness with these products [361,365], information on clinical response has only been assessed by practitioner surveys [366,367]. Mechanisms for positive effects of PSGAGs are reduced PGE₂ production from inflamed synoviocytes [368], and increased hyaluronan production [369,370]; however PSGAG caused decreased proteoglycan content in normal cartilage explants [371]. Overall, PSGAG and pentosan sulfate have moderate evidence of some disease modifying effects in experimental OA and *in vitro*; however, more research needs to be performed to assess intra-synovial concentration after injection and to objectively assess response to treatment in clinical patients.

– Viscosupplementation: Hyaluronic acid

The concept of viscosupplementation was hypothesized as a treatment approach [372,373] to counteract degradation of hyaluronic acid (HA) by hyaluronidase and reduced synthesis in OA [374]. Like the use of glucosamine, meta-analyses of intra-articular hyaluronic acid have had conflicting results [309,337,375], leading to alteration of previous recommendations [274]. However, the most recent meta-analyses have found significant reduction in pain intensity and improved function [376,377]. These meta-analyses used criteria to select low bias, placebo controlled trials [376,377] and contrast to the earlier studies that did not use rigorous selection criteria [375].

Hyaluronic acid (HA) is more effective at reducing pain in late OA [309,332]. Corticosteroid injection was superior to hyaluronic acid injection initially (up to 4 weeks), but HA improved pain relief at weeks to 26 weeks [309] and at 1 year compared to a combination of corticosteroid

and HA [332]. Mechanisms for positive treatment effects of HA are chondroprotection, enhanced of proteoglycan synthesis, anti-inflammatory and mechanical action [378,379]. Hyaluronic acid binds to CD44, inhibiting IL-1 β expression with subsequent reduction in MMPs [380,381], inhibition of PGE₂ and other down-stream inflammatory mediators [382], enhanced proteoglycan synthesis [383], inhibition of chondrocyte apoptosis [381,384]. Additionally, HA is a viscous substance that lubricates the synovium and cartilage resulting in decreased friction [385]. The degree of proteoglycan synthesis [386] and reduction in inflammation [387] is linked to the molecular weight of HA *in vitro*. HA with a molecular weight of greater than 1x10⁶ Daltons reduces NF κ B levels compared to low molecular weight HA [388] and clinical trials in humans have found that high molecular weight HA (average 6x10⁶ Daltons) reduces symptoms of OA compared to low molecular weight HA (average 0.5-0.75x10⁶ Daltons) [389-391]. In contrast, cross linked HA at very high molecular weights (23x10⁶ Daltons) increases the risk of local adverse events [392].

Veterinarians commonly administer hyaluronic acid formulations intravenously, in addition to or instead of intra-articular [310]. Both routes had an effect on lameness induced by Freund's adjuvant [393], but treatment of OA induced using a carpal osteochondral fragment model found that intravenous administration decreased lameness, PGE₂ concentrations and synovial inflammation compared to placebo treated controls [394], whereas a decrease in cartilage fibrillation was only observed with intra-articular administration [361]. Hyaluronic acid is quickly eliminated from both the plasma and the synovial fluid after intravenous or intra-articular administration [395].

Both anti-inflammatory and chondroprotective effects on equine cartilage have been found *in vitro* [295,316,318,326], which are possible at very low (0.2 μ g/mL) concentrations to reduce PGE₂ production by synoviocytes [368] and proteoglycan release by metacarpal cartilage [396]. Like glucosamine, the preparation of hyaluronic acid could alter HA treatment effect. For example, non-animal stabilized hyaluronic acid had no reduction in lameness or joint effusion compared to placebo [397]; intra-articular high molecular weight HA reduced lameness compared to low molecular weight HA [393]; and an intravenous preparation of HA that also contained chondroitin sulfate and N-acetyl-D-glucosamine caused worsening of radiographic scores and bone edema [398]. HA has evidence for anti-inflammatory and disease modifying

effects in equine OA; however, the formulation needs consideration and direct comparison of intra-venous and intra-articular administration with high or low molecular weight products should be performed in the future.

Treatment of end-stage OA

There are key differences in the treatment of people and horses who have end stage OA: in people surgical arthroplasty is performed to restore joint function, while in horses certain joints can undergo arthrodesis (joint fusion) to restore limb function or as a salvage procedure. The main indications for arthroplasty or arthrodesis in both humans and horses are severe pain/lameness and severe radiological lesions [399]. There are anatomical limitations as to what joints can undergo surgical treatment for end-stage OA. In humans the knee, shoulder, hip and tibiotarsal joints can be replaced by prosthesis; in horses surgical arthrodesis has been described for the distal and proximal interphalangeal joints, the distal tarsal joints, the metacarpophalangeal joint and the carpus [400]. For human knee replacement 36-89% of patients return to sport, however it is at a reduced level of intensity [401] and 5.6% [402] of patients have worse outcome measures after 6 months with 3.8% [403] requiring additional surgery [404]. Patients having total knee arthroplasty are becoming increasingly younger [405], while life expectancies continue to increase [406]. Given that the annual failure rate of total knee arthroplasty is 0.5-1%, the probability of arthroplasty lasting 10 years is 90-95% and 20 years 80-85% [407]; therefore, the number of revision surgeries is expected to rise and with increased complications [408].

For horses, only arthrodesis of the proximal interphalangeal joints, distal tarsal joints or carpometacarpal joints enable horses to return to athletic function [400]. Eighty-one to 85% of horses with front limb and 89-95% of horses with hind limb proximal interphalangeal surgical arthrodesis returned to previous levels of use [409,410]; however, horses that performed at high levels had lower success (25% and 73% for fore limb and hind limb, respectively [411]). Fifty-nine to 71% of tibiotarsal joints with surgical arthrodesis returned to previous level of function [412,413]. Metacarpophalangeal and pan-, inter- or radio-carpal arthrodesis procedures are limited to high value breeding animals because they result in mechanical lameness [414]. All surgical procedures incur significant cost and the risk of post-operative infection in internal fixation is approximately 10%; as a result, many horses who are candidates for arthrodesis

succumb to wastage [415]. Given that treatment of end-stage OA is fraught with complication, expense and physical limitations after surgery, treatments to halt or reverse OA continue to be the ultimate goal for OA researchers and clinicians.

Summary

OA is of significant economic and social burden for both humans and horses. The disease process is initiated by separate or combined etiologic factors that have a common inflammatory mechanism. Once the disease is initiated it is propagated by inflammatory events in a similar manner despite the initiating factors. Disease progressions is slow, but marked by significant pain and loss of joint function as all joint tissues degenerate and lose ability to maintain joint homeostasis. The inability of the joint to repair has made treatments to prevent, halt or regenerate tissue destruction the herculean task of OA clinicians and researchers.

The review of pharmacologic drugs to treat human and equine OA has shown the variation in efficacy that can occur in both symptom relieving and disease modifying effects. All of the therapies described have had an ameliorating effect on inflammatory processes that occur in OA. However, the therapies discussed have significant limitations on their ability to be anti-inflammatory as well as modify disease. For example, NSAIDs only decrease inflammation, and although corticosteroids are anti-inflammatory, they have deleterious effects on articular cartilage. DMOADs like glucosamine/chondroitin sulfate and hyaluronic acid have mechanisms to complete both actions; however, there is significant controversy in their ability to be sufficiently symptom relieving or disease modifying. These limitations have fueled clinicians and researchers to look beyond pharmaceuticals and investigate biologic therapies for OA. The great need to find a solution is evidenced by the limitations on both humans and horses faced by end-stage disease.

CHAPTER 2

Investigation and Role of Biologic Therapies for Osteoarthritis

Osteoarthritis (OA) is a complex disease process that involves all tissues of the joint in a cycle of inflammation and tissue degradation that is irreversible [7]. Treatments for osteoarthritis mainly decrease symptoms of OA and drugs that were historically thought of as disease-modifying (DMOADs) have recently been down-graded due to insufficient levels of efficacy [274]. Additionally, there are no treatments with a truly anti-inflammatory effect that promote anabolism of the cartilage matrix. Another issue with the pharmacologic therapies commonly used in OA is that equine athletes cannot compete as sport or racehorses when they have therapeutic, or sometimes trace, plasma concentrations of pharmaceuticals [299,303,304].

Biological therapies use the body's own immune system or soluble cytokines to ameliorate a disease process [416,417]. They contain enhanced quantities of biological response modifiers, which are normally produced in the body at low levels and include stem cells, anti-inflammatory cytokines, growth factors, or a combination [418]. Biological therapies may be recombinant proteins, autologous (self) or allogeneic (other) blood or tissue, monoclonal antibodies, gene therapy or drugs that condition a primary biological effect [416]. Biological therapy is closely linked with regenerative medicine, which focuses on the ability of biologic therapies to regrow, repair or replace damaged cells or organs [419]. In OA the goal of biologic therapy and regenerative medicine is to alter the inflammatory cycle of the disease, regenerate damaged tissues or, ideally, both.

The horse as a model for OA

There are numerous *in vitro*, *ex vivo*, and *in vivo* models of OA developed for testing specific aspects during pathogenesis or treatment of the disease. *In vitro* models provide a cost and time effective method for examining specific responses to therapies. For example, *in vitro* models exposed to corticosteroids were able to show reduced proteoglycan and collagen II synthesis of chondrocytes and that cartilage degradation was primarily due to proteoglycan depletion

[291,315,318,330,420]. *In vitro* models can be a monolayer culture of one cell type, co-culture, three-dimensional culture or an explant of articular cartilage or synovium. Not only can the cell type vary, but also the stimulation used to produce changes that are degenerative and/or inflammatory. IL-1 β and/or TNF α are the most common inflammatory cytokines used for *in vitro* models given their primary role in the development of down-stream inflammation and cartilage degradation [250,421]. These cytokines are often applied at supra-physiologic levels; however, there are models that expose three-dimensional chondrocyte cultures or cartilage explants to low levels of inflammatory cytokines for a prolonged (~7 day) period to bring about cartilage catabolism similar to naturally occurring disease [422-426]. Application of mechanical damage to cartilage explants has also been used to induce low-grade inflammation [76,427]. While *in vitro* models are valuable for investigating specific pathways altered by a therapeutic intervention, they are limiting when understanding global therapeutic efficacy. As described in chapter 1, OA initiation and progression is multifactorial and involves all of the joint tissues with complex cytokine-cell and cell-cell interactions. Therefore, *in vivo* models give a more accurate understanding of how a therapy interacts under complex disease conditions.

Like *in vitro* models, there are many different *in vivo* experimental models of OA. These experimental models allow induction of joint inflammation or cartilage degradation under controlled conditions as opposed to the complex and variable factors that incite OA in clinical patients [7]. OA can be induced *in vivo* by creating joint instability [428-430], intra-articular trauma to the subchondral bone [180] and/or cartilage [179,431,432], animals that naturally develop OA [433], transgenic models [434] or through intra-articular injection of cytokines or other inflammatory stimulant [435-437]. It is important for the researcher to choose the model that best represents the aspects of disease that are being altered with therapy, particularly with biologic therapies that are multi-modal and are affected by disease environment. For example, anterior cruciate ligament transection/ medial meniscectomy model was used to test the effects of bone marrow derived stem cells (BMSCs) in goats and concluded amelioration of OA was due to reduced articular cartilage degeneration, subchondral bone sclerosis and osteophytes [438]. However, it is now known that the main effect of BMSCs in this model is to regenerate intra-articular tissues; therefore, the improved hallmarks of OA were mainly due to increased stabilization compared to the control. Similarly, intra-articular injection of a cytokine or

inflammatory stimulant such as LPS causes intense synovial and chondrocyte inflammation that is supra-physiologic compared to naturally occurring OA [253,435,439]. However, these models can be used for a clear distinction of the effect of anti-inflammatory therapies prior to testing in a model with lower levels of inflammation.

Horses represent a realistic model species for human OA due to their size, lifestyle and the degree of OA drugs that have already been tested under different *in vivo* conditions [440]. Compared to other model species such as rodents and rabbits, commonly used to test OA therapies, equine joint anatomy is more similar to humans in terms of size and articular cartilage thickness. For example human articular cartilage is approximately 2.2-2.5mm with similar, 1.75-2mm, thickness in horses [441]. Additionally, enough synovial fluid can be collected via arthrocentesis to allow a range of biochemical assays and fluid analysis. Like humans, horses suffer from naturally occurring disease and their use as athletes requires OA treatment and return of joint function, which is a treatment goal for human OA [15,16,25]. Objective and subjective return of joint function can be determined through lameness examination with grading, inertial sensors or force plate meters, joint flexion tests or goniometry [442-445]. The horse also provides an animal model with similar degrees of life-style variation to people, so that retrospective analysis of treatments can be performed in a “real-world” setting prior to investigation of a treatment for use in people. Their ability to perform high intensity exercise also makes them a sensitive model for the effects of symptom-modifying treatments on cartilage health; for example, the negative effects of corticosteroids on cartilage metabolism were highlighted in an equine model that was treadmill exercised [314,324]. Furthermore, due to the value of horses as competition athletes, testing of various OA drugs has already been performed so consistently predictable *in vivo* models have been developed for testing symptom and disease modifying effects of therapies, while *in vitro* models are established for investigating underlying mechanisms of action [440,446].

Experimental *in vivo* models of equine OA have been developed with predictable levels of inflammation, cartilage matrix degradation and lameness [440]. The most widely used models are the carpal osteochondral fragment model, developed at Colorado State University (CSU), and the LPS induced synovitis model. The CSU model involves arthroscopic creation of an 8mm osteochondral fragment on the distal dorsal aspect of the radial carpal bone with consistent tread-

mill exercise and treatment initiated 2 weeks after surgery [27]. This model has been used to investigate the effects of intra-articular or systemic pharmaceuticals [301,361,365,394,398] as well as biologic therapies including stem cells [447], autologous conditioned serum [448] and gene therapy [449]. Additionally, the pathological response over time has been analyzed and inflammation levels are low as to be expected with naturally occurring OA [27]. The main limitations are that many horses exhibit intermittent (grade 1 AAEP) lameness and the degree of inflammation may be too low to assess a binary result for biologic anti-inflammatory therapies [447]. The LPS induced synovitis model involves intra-articular injection of 0.5ng LPS per joint, which is the level determined not to cause systemic endotoxemia [436]. Like the CSU model, it has been used to test the anti-inflammatory effects of intra-articular and systemic pharmaceuticals [288,450-454] as well as biologics [455]; and the pathologic events over time have been characterized [253,439]. The main advantage for testing anti-inflammatory therapies is that it produces obvious (grade 2-3 AAEP) lameness that is related to the degree of synovial inflammation, therefore the anti-inflammatory capacity of therapies can clearly be determined [436,451]. The synovitis lasts for up to 72 hours and horses recover without lasting effects [253,436], unlike the CSU model that necessitates euthanasia. As a result, the LPS synovitis model is a logical and humane choice to initially test anti-inflammatory joint therapies.

Biological therapies for equine OA

Regulation and investigation of biologic therapies in animals

Animal biologic and most cell based products meet the Food and Drug Administration's (FDA) definition of a drug because they are intended to treat, control or prevent a disease or other condition (U.S.C.321(g)(1)(B)&(C)). As a result, no biologic nor most cell based products can be legally marketed unless they have gone through pre-market review of experimental data to ensure that the product is safe, effective and high quality [456]. For the veterinary industry, this constitutes a new animal drug application (NADA) (21 U.S.C. §§ 331(a) and 360b(a)). There are currently no animal cell-based products or biologic products other than vaccines, toxoid and anti-bodies that are FDA approved [457]. This is because the definition of efficacy necessitates that the drug's strength, quality, and purity are maintained from batch to batch. There is inherent variation with biologic therapies as the levels of cytokines, growth factors, stem cell activity or

other biological response modifiers vary with multiple factors that include individual, diurnal variation, environmental stress and processing procedures [458-462]. To avoid this limitation, specific devices used to create biologic therapies by the user can be sold and marketed because pre-market approval of veterinary devices or registration of device manufacturers is not required by the FDA [463]. As a result, there are a plethora of devices and kits currently marketed to produce autologous conditioned serum and platelet rich plasma for equine OA.

Mesenchymal stem cells have been used successfully for the treatment of various equine musculoskeletal diseases including tendinopathy [464-466], intra-articular soft tissue injury [467] and cartilage regeneration [468,469]. In June 2015 the FDA released guidelines for the veterinary industry on cell-based products [1]. The guidelines defined types of cell therapies to clarify what products would likely need NADA prior to legal marketing. The guidelines distinguished between autologous, allogeneic and xenogeneic therapies. Autologous cell therapies for non-food producing animals were further divided into two categories: type I and type II. Type I are cell-based therapies that are more than “minimally manipulated” i.e. have processing to alter their relevant biological characteristics, are “non-homologous” i.e. use donor cells to replace recipient tissue with different basic functions, have an effect that is dependent on cell metabolic activity or are combined with another article or device. Examples of type I include any stem cells expanded in culture. In contrast, type II autologous cells are minimally manipulated, used in a homologous manner and are used without any additives or other devices. An example of type II would be isolated non-expanded chondrocytes used to fill an articular cartilage defect.

Whereas previously there had been no limitations on the development or use of cell based therapies for non-food animals [470], now to legally market cell based therapies that are allogeneic, xenogeneic, or fall in the type I autologous category, pre-market review through the New Animal Drug Application (NADA) pathway is needed to demonstrate safety, effectiveness and manufacturing quality [1]. The NADA pathway begins with opening an Investigational New Animal Drug (INAD) file after sufficient pilot data using *in vitro* or *in vivo* testing on research animals has been performed (which does not need FDA involvement), prior to starting investigation on client-owned animals. However, manufacturers can deliver products for clinical trials without an INAD if they submit notice of claimed investigational exemption to the FDA

and follow specific distribution and labeling requirements. Many cell-based products are currently used in non-food animal veterinary practice without FDA involvement because they are not marketed, or promoted as being safe or effective, or sold into interstate commerce.

Non-cellular biologic therapies for equine OA

– Platelet rich plasma

Platelet rich plasma (PRP) is made by concentrating platelets in plasma obtained from whole blood using a kit marketed as a “device”. The minimum platelet concentration that defines human PRP is >1 million platelets per μL [471]; however there are no minimum platelet concentrations or fold increase over systemic platelet count for equine PRP. When platelets in PRP are activated by the disease environment or prior to injection through the use of CaCl_2 , bovine thrombin or a combination, the alpha granules release growth factors and cytokines. The two main growth factors in PRP are PDGF and TGF- β , however there are also VEGF and IGF-1 [458]. Human studies have shown that TGF- β 1 and IGF-1 stimulate extra cellular matrix synthesis from chondrocytes [472,473] and IGF-1 decreases synovial inflammation [474]; however TGF- β 1 has undesirable effects on the synovium including increased leukocyte infiltration, synovial fibrosis and osteophyte formation [475]. Another mechanism of action is PRP stimulation of synoviocytes to produce hyaluronic acid [476], which may be more important in early OA as no differences were found between people treated with PRP or hyaluronic acid [477], except if cartilage degeneration was present [477,478]. A potential limitation of PRP is that inflammatory cytokines including IL-1 β , IL-6 and IL-8 have been found using different preparation techniques with human blood [479,480]. However, inflammatory cytokines are related to leukocyte content and can be reduced by leukocyte depletion filtration [479].

PRP use for equine OA has shown some success in clinical studies; however, production and activation techniques differ. PRP improved lameness and effusion scores in a pilot study of 4 horses [481], PRP with pre-activation of platelets via freeze-thaw improved lameness associated with distal interphalangeal joint OA compared to a saline control [482], while a larger study for naturally occurring equine OA using a derivative of PRP that undergoes a 2 step process to increase platelet concentration and cytokines also found improved lameness scores [483]. The main limitation is that there was either little or incomplete analysis of the growth factor and

cytokine profiles of the products tested. This is an issue because of high variability associated with preparation system [484], platelet activation [485] and individual horse factors [458] that could affect the clinical response to PRP treatment. Leukocytes are also concentrated during equine PRP processing, and the quantity of leukocytes affects the ability of PRP to ameliorate inflammation from equine synoviocytes [484]. Additionally, there is variation in the ability to concentrate platelets of available systems currently used for horses, which influences growth factor levels [486]. The largest source of variation lies with activation method; although thrombin-activated PRP has higher levels of growth factors, when injected into healthy metacarpo-/metatarsophalangeal joints it caused joint effusion [487] with increased synovial fluid TNF α and IL-6 [485]. Further research to ascertain the efficacy of PRP products derived from various systems needs to be performed prior to widespread use for OA, and equine clinicians need to be guarded against the use of bovine thrombin for intra-articular use.

– Autologous conditioned serum

Autologous conditioned serum (ACS) uses whole blood that is treated with chromium sulfate treated glass beads to increase production of anti-inflammatory cytokines, such as IL-1ra, by direct physico-chemical induction [488]. Like PRP, in veterinary medicine ACS is made using a kit sold as a veterinary “device”. The initial description of ACS production measured a 140-fold increase in IL-1ra, which is a protein that binds to the IL-1 receptor thus antagonizing the inflammatory effects of IL-1 [488]. Additionally, the anti-inflammatory cytokines IL-10 and IL-4 were increased approximately 2-fold with no increase in the inflammatory cytokines IL-1 β and TNF α [488]. Like PRP, preparation technique [461,489,490] and individual variation [462] alters the bioactive composition of ACS. The two main systems of ACS production for horses are IRAP I (Dechra Veterinary Products) and IRAP II (Arthrex). IRAP II achieves a higher IL-1ra:IL-1 ratio than IRAP I [461]. Pro-inflammatory cytokines IL-1 β and TNF α are also increased with ACS preparation [461,489]; therefore, the ratio of anti-inflammatory to pro-inflammatory cytokines may be important for therapeutic effect [461]. Horses that have undergone surgical stress produce ACS with reduced IL-1ra and TGF- β levels that are lower with higher levels post-operative systemic inflammation [462]; therefore, the timing of ACS collection may be important in horses undergoing surgical treatment.

Clinical results for the treatment of equine OA with ACS have been promising, however the exact mechanism of action remains incompletely understood in both the veterinary and human fields. Treatment of a carpal osteochondral fragment model of equine OA with IRAP I injected 4 times at weekly intervals found improved lameness, synovial thickness and cartilage fibrillation compared to saline treated controls [448]. The injection frequency of ACS is likely important. Horses with arthroscopically defined naturally occurring OA treated with 3 injections of IRAP II at 2 day intervals had significantly lower levels of IL-1 β , biomarkers of cartilage degradation and IL-1ra 42 days after treatment initiation compared to horses injected at 7 day intervals [491]. Despite clinical improvements, *in vitro* studies have not shown chondroprotective effects; ACS did not alter proteoglycan release or production in OA human articular cartilage explants compared to serum [489]. Furthermore, although ACS (IRAP II) increased IL-1ra and IGF-1 in equine cartilage explants treated with IL-1 β , there was no significant difference in MMP-3 production and proteoglycan loss or synthesis between ACS and autologous serum treated samples [490]. The ability of ACS to provide chondroprotection and mechanisms of action remain incompletely understood so efficacy is currently based on improvement in clinical signs and symptoms in both horses and people.

Introduction to mesenchymal stem cells

Defining stem cells

Stem cells are adult or embryonic in origin (Fig. 2.1). Embryonic stem cells (ESCs) have telomerase, allowing repletion of chromosomal length that equates to an immortal phenotype [492]. Adult stem cells do not have telomerase so undergo senescence in 30-40 population doublings; however, this gives them clinical advantages such as reduced tumorigenicity when used *in vivo* [493,494]. Traditionally, embryonic cells were defined as totipotent or pluripotent i.e. able to differentiate into any cell of the body and extra-embryonic tissues or only cells of the body, respectively. In contrast, mesenchymal stem cells (MSCs) isolated from mesenchymal tissues of an adult, were defined as multipotent i.e. only able to differentiate into cells of mesenchymal origin. However, MSCs have been found to differentiate into non-mesenchymal lineages indicating the possibility of transdifferentiation and highlighting stem cell plasticity [495,496]. Human MSCs have been defined by three main criteria: they are 1) plastic adherent,

2) have specific surface “cluster differentiation” (CD) antigen expression of CD105+, CD73+, CD90+, CD34-, CD45-, CD14- CD79 α -, MHCII-, and 3) have multipotent differentiation potential [497].

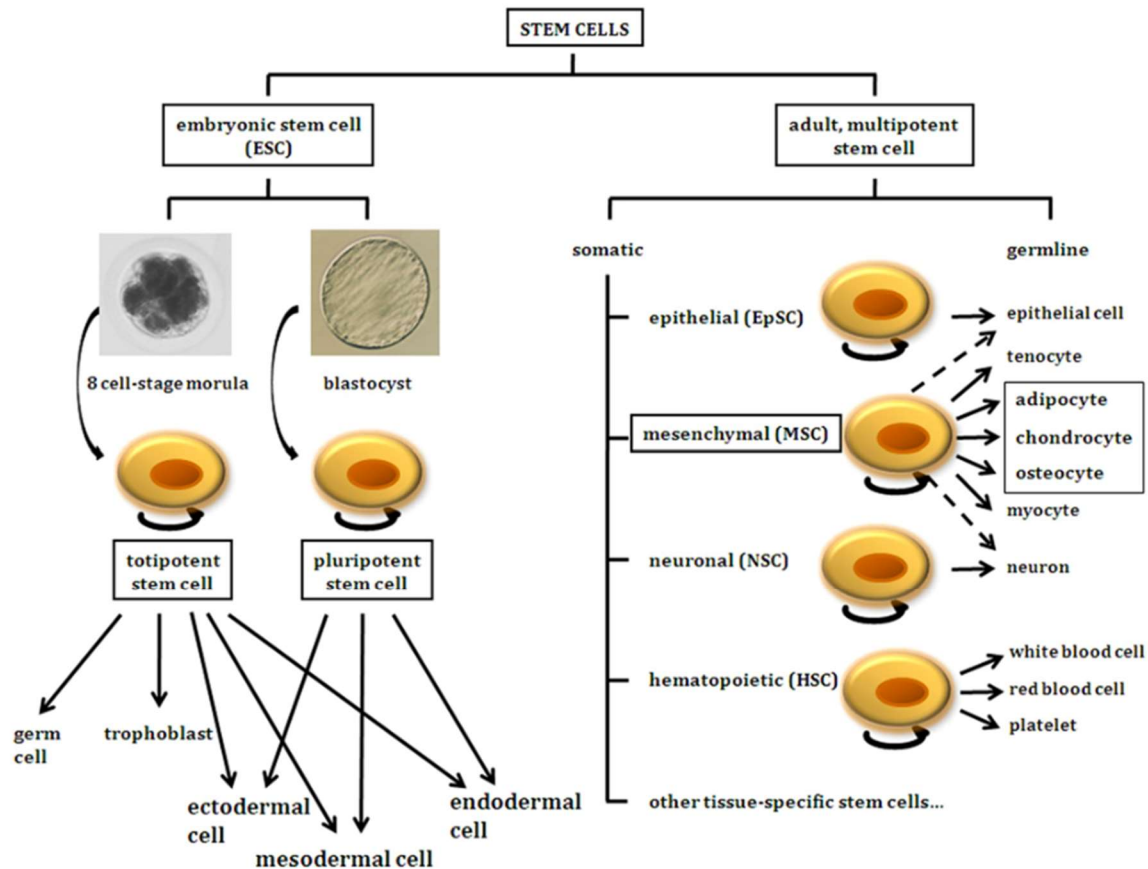


Figure 2.1 – Stem cell types and differentiation potential

The classification of stem cells based on tissue origin and differentiation abilities are being challenged by the ability of MSCs to transdifferentiate [498]^{iv}

Equine MSCs (eMSCs) have not been formally defined, however there is agreement on their ability to be plastic adherent and undergo tri-lineage differentiation, with less consensus on CD marker expression [498]. CD markers are surface or intra-cellular antibodies expressed at different stages of differentiation and are commonly assessed by flow-cytometry, but lack of cross-reactivity between human and equine anti-bodies made quantitative polymerase chain reaction (qPCR) a more accurate method of detection for equine CD markers if cross-reactivity

^{iv} Reprinted from Theriogenology, 75/8, De Schauwer C, E Meyer, GR Van de Walle and A Van Soom, Markers of stemness in equine mesenchymal stem cells: A plea for uniformity, 1431-1443, Copyright (2011), with permission from Elsevier.

was unknown [499]. Markers that have been used to define eMSCs via flow-cytometry are CD29+, CD44+, CD90+, CD105+, CD14-, MHC1+, CD79 α -, MHCII-. These have also been detected via RT-PCR, in addition to the pluripotency markers Oct-4, Sox-2 and Nanog that control differentiation [498].

Sources of MSCs

MSCs have been isolated from every adult tissue, however their exact cellular origin is debated [500] with some authors theorizing they come from a vascular pericyte population given a similar transcriptome and differentiation ability [501,502], and others citing origin in neuro-epithelium with a transition under genetic control [503,504]. It is most likely that the heterogeneity of MSCs is because *in vivo* niche varies, for example, heterogeneity in differentiation ability, stem cell marker expression and activity found between stem cells of the same bone marrow aliquot could be due to MSCs arising from the endosteal, peri-vascular or stromal niche [505-507].

Bone marrow-derived MSCs (BMSCs) and adipose-derived MSCs (AdMSCs) are the most common sources for clinical use. In horses, BMSCs are harvested from the sternum or ileum [508-510] and AdMSCs from adjacent to the tail head [511]. BMSCs derived from the sternum and ileum have no difference in differentiation ability, markers of stemness [509], cell counts and growth rates [512], number of BMSCs or ability to form colony forming units [513]. However, a study using horses ≤ 5 years old found that ileal BMSCs had slightly more population doublings per 24 hours than sternal BMSCs [513], but sternal aspirates had a higher yield of total BMSCs than ileal aspirates in 13-year-old geldings [514].

Equine BMSCs and AdMSCs show inherent differences in differentiation potential, senescence and immunophenotype/ stem cell markers. Equine BMSCs (eBMSCs) have increased expression of osteogenic markers compared to AdMSCs when stimulated to undergo osteogenic differentiation, but there is no difference in calcium deposition and ALP activity [515], another study found no differences in tri-lineage differentiation between AdMSCs and BMSCs [516]. Immunophenotype of equine BMSCs and AdMSCs is similar with no difference in expression of CD105, CD90, CD44 or MHCII, however AdMSCs had increased CD34 [516]. Overall, AdMSCs have increased proliferation capacity [515,516]; however, this depends on the passage

number and amount of population doublings because there was little difference in initial passages ($\leq 4^{\text{th}}$ passage), but after passage 9 BMSC doubling time significantly increased with senescence at 30 population doublings ($\sim 10^{\text{th}}$ passage), whereas AdMSCs senesced at around 70 population doublings [517]. Osteonectin, a genetic marker for osteogenic differentiation, was expressed in both AdMSCs and BMSCs as they underwent senescence and was increased at passages 3-4 in BMSCs [517]. Therefore, use of low passage BMSCs may be indicated to reduce the chance of osteogenic differentiation, however there are no reports of heterotopic calcification in horses attributed to BMSCs treatment in studies using serial ultrasound [465,466,518]. BMSCs remain the MSC source with the widest use and most research for equine orthopedic disease with no direct comparisons to AdMSCs for efficacy in specific disease conditions.

There are inter-donor differences in MSC ability to differentiate, proliferate and in stem cell markers [500]. Inter-donor variation was a higher source of variation than the difference between stem cell sources in one study of equine stem cells [519]. These differences have also been shown in human [520] and murine [521] BMSCs. Such differences have been attributed to donor age [522-525], minor differences in sample processing or collection [508,512,526]. Donor can also have significant therapeutic implications during allogeneic MSC treatment. Variation between healthy human BMSC donors (18-45 years old) in a rodent model of spinal cord transection was responsible for variation in axon growth and the degree of recovery in behavioral tests [527]. However, specific age-matched studies have not been performed so the scope of phenotypic or therapeutic inter-donor variation of BMSCs is not fully understood.

General therapeutic properties of MSCs

MSCs have regenerative, anti-inflammatory, immunomodulatory and trophic functions [528]. As a result of the multi-faceted nature of stem cell function, they are being investigated in the treatment of a wide range of diseases. As of February 2017, the U.S. National Institutes of Health had 102 open clinical trials using BMSCs to treat diseases from systems including neurological, ophthalmic, orthopedic, cardiovascular, urogenital and renal [529]. MSCs are often administered directly to the site of injury or location of desired response; however, homing of human MSCs to injured tissue under the control of trophic factors has been demonstrated in

murine radiation induced injury [530,531], murine long bone fractures [532,533] and traumatic central [534] and peripheral [535] nervous system injury in rats. Once at their location, MSCs respond to environmental queues to guide their functional effects and act on endogenous MSCs, which may play a greater role than those administered [504,536].

The regenerative capacity of MSCs was the first to be investigated, mostly due to the finding of tri-lineage differentiation, and is applied to *in vitro* and *in vivo* tissue engineering and regeneration. There are two approaches to MSC derived tissue regeneration: 1) application of cells that will remain and differentiate to form part of the recipient tissue, 2) application of cells that will have trophic effects on endogenous stem and immune cells to speed endogenous tissue repair. The second mechanism is likely the main mechanism of tissue regeneration and has been demonstrated in large animal models. For example, the addition of peripheral blood derived MSCs to PRP did not improve histological or biochemical healing of sheep tendon injury [537], and the integration of labeled equine BMSCs into tendon is low [538]. Furthermore, injection of culture media conditioned by MSCs derived from horse amniotic membrane into naturally occurring tendonitis decreased tendon re-injury rate and had a similar clinical and ultrasonographic course of healing as when whole cells were injected [539,540]. The persistence and regenerative effects at a delivery site are dependent on the stem cell niche encountered. The stem cell niche is the microenvironment that interacts with the stem-cell. Key niche factors include immune/ inflammatory cells, soluble factors, signaling receptors, biomechanical forces and extra cellular matrix interactions [501,504,541]. The importance of niche factors is exemplified by the relationship between macrophages and MSCs. Regeneration of myocardial infarcts in a murine model occurs with direct autologous BMSC injection; however, there was no effect of MSCs when macrophages were depleted [542]. Additionally, interaction of human and equine BMSCs with tensile forces prompts differentiation into tenocytes with no other stimulation [543-545].

Another key function of MSCs is their ability to reduce inflammation via immunomodulation. MSCs need to be induced into an anti-inflammatory phenotype by the stem cell niche, which has been termed “cytokine licensing”. $\text{IFN}\gamma$ is the most important factor to induce an anti-inflammatory state in MSCs and is synergistic with $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-17 [496]. The importance of prior activation to an anti-inflammatory state has been seen clinically by reduced

efficacy when MSCs are administered to patients with concurrent systemic corticosteroids [546], compared with those who are refractory to corticosteroid treatment for graft vs. host disease [547]. Also, human and murine BMSCs have increased anti-inflammatory efficacy in murine models of acute colitis [548] and graft vs. host disease [549] when pre-treated with IFN γ . Conversely, MSCs can increase inflammation when they are used in a non-inflamed niche, this was seen most recently in horses when acute synovitis occurred with injection of cord-blood MSCs alone, but was reduced when the inflammatory agent LPS was injected concurrently [455]. A higher degree of anti-inflammatory activity was seen in a murine hepatitis model when murine BMSCs were pre-treated with IFN γ in combination with TNF α and IL-17 than when used alone [550]. These findings have led to an understanding that in high levels of inflammation MSCs have high anti-inflammatory efficacy, however efficacy decreases as the level of inflammation decreases (Fig. 2.2) [551]. When BMSCs become “cytokine licensed” they have increased production of TSG-6, IL-6 and PGE $_2$ [4,214,552-555]. Pre-stimulation to an anti-inflammatory phenotype may decrease some of this variation and, in doing so, increase the reliability of MSC therapies.

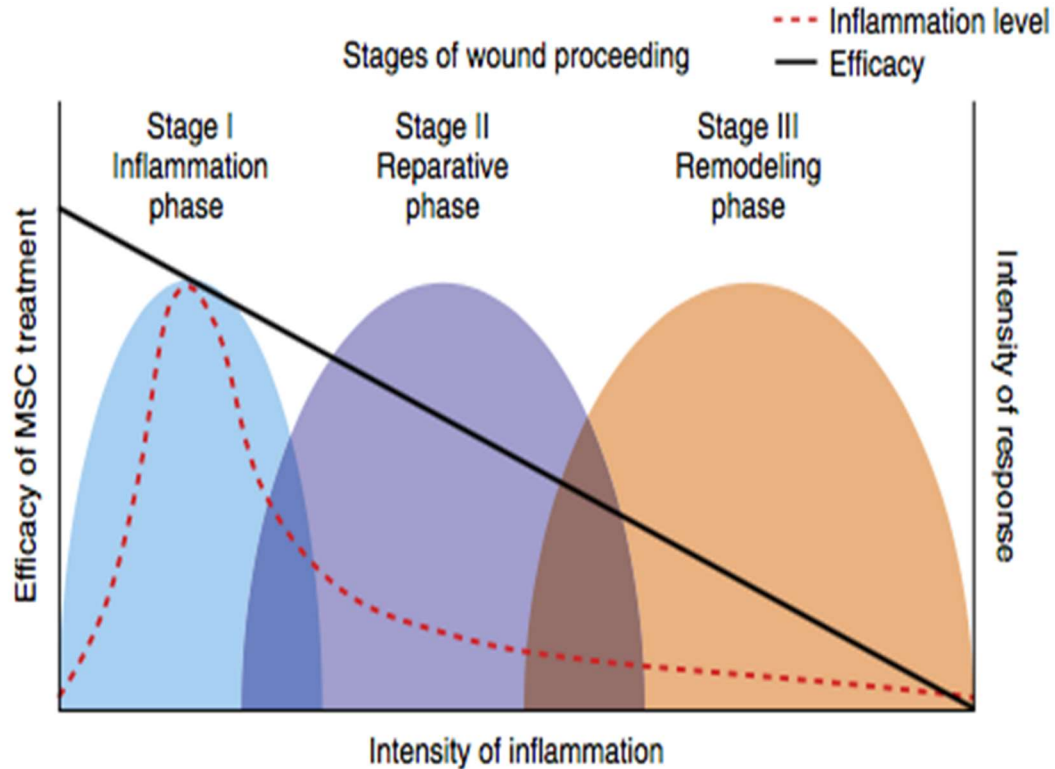


Figure 2.2 – Cytokine licensing of MSCs

The efficacy of immunomodulation and reduced inflammation changes with the inflammatory niche [496]^v

MSCs mainly use paracrine mechanisms to alter the inflammatory behavior of cells of the innate and adaptive immune systems, thereby having anti-inflammatory effects that act through an intermediate [556]. For example, BMSCs cause a change in phenotype of macrophages stimulated with LPS from a $\text{TNF}\alpha$ producing inflammatory M1 state, to an anti-inflammatory M2 state that produces increased IL-10, decreased $\text{TNF}\alpha$ and IL-12, and promote T cells to produce less pro-inflammatory cytokines [4,214,557]. They can also modulate T cells, dendritic cells and neutrophils by reducing proliferation, chemotaxis and oxidative burst [496,558]. A variety of soluble mediators are released by MSCs for their anti-inflammatory and immunomodulatory effects and vary with species [559], disease micro-environment, and source [553]. PGE_2 , TSG-6 and IDO are the most commonly found soluble factors that human BMSCs release to modulate inflammation [496]. PGE_2 is the key soluble mediator used by human BMSCs to modulate the inflammatory activity of immune cells, resulting in reduced $\text{TNF}\alpha$ and

^v Reprinted from Nat Immunol, 15/11, Wang Y, X Chen, W Cao and Y Shi, Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications, 1009-16, Copyright (2014), with permission from Nature Publishing Group.

increased IL-10 production [558]. Additionally, BMSC derived PGE₂ altered the phenotype of macrophages in a dose-dependent manner [4] and was the main mediator from AdMSCs to reduce the production of inflammatory cytokines from human chondrocytes and synoviocytes [209]. In a variety of *in vitro* models BMSC efficacy has been reduced when PGE₂ was blocked in a dose-dependent manner [4,209,560]. Some anti-inflammatory mediators are directly produced by MSCs, such as IL-1ra and IL-10. However, these factors were only found in a subgroup of MSCs after clonal selection [561,562].

MSCs can be used therapeutically in an autologous, allogeneic or xenogeneic manner.

Autologous therapy uses MSCs derived from the tissue of the recipient, in contrast to allogeneic treatment that uses a different donor tissue than that of the recipient, but is the same species.

Xenogeneic treatment is the use of tissue derived from a donor of a different species. Currently autologous use is most common in human and equine medicine as the perceived risk of immune rejection is lower, although this remains unsubstantiated and the number of clinical trials using allogeneic MSCs is increasing [529]. Allogeneic MSCs can decrease proliferation of T cells, alter the phenotype of macrophages and cause reduction in inflammatory cytokines in humans and horses in a similar manner to autologous MSCs [563-566]. In a study that used allogeneic hematopoietic MSCs in humans, no antibodies to the MSCs were found, but there were antibodies to the fetal bovine serum that the cells were cultured in [567]. In contrast, use of allogeneic BMSCs and AdMSCs in horses caused the detection of alloantibodies [568]. MSC's are classified as lacking MHC II, however some conditions can cause MHC II expression [569], if this occurs and donor and recipient MHC II and MHC I are mismatched, there is a higher chance of immune reaction in mice [570] and horses [569]. Despite these findings allogeneic MSCs have been successfully used *in vivo* without immune rejection in equine tendinopathy [571] and synovitis [455]. Xenogeneic use is less common and reserved for experimental models, for example to test the effect of human MSCs in an animal model of diseases, including OA [572]. Xenogeneic BMSCs have been used in healthy joints of horses and caused mildly increased joint effusion and synovial inflammatory cytokines compared to autologous or allogeneic BMSCs [573]. Allogeneic or xenogeneic preparation of MSCs provides promise for off-the shelf treatment, as currently propagation of autologous MSCs is prolonged (~4 weeks); therefore, does not allow clinicians to take advantage of the inflammatory niche in the acute to subacute period after injury. Additionally, allogeneic use would allow harvest from a younger or

non-diseased donor and may increase therapeutic efficacy. For example, MSCs from human patients with advanced OA had reduced chondrogenic activity [574]. Understanding the biological and therapeutic limitations of autologous, allogeneic and xenogeneic therapies are important when optimizing MSC based therapies.

Advantages and status of mesenchymal stem cells for osteoarthritis

MSC characteristics that give them therapeutic potential in OA

The benefit of MSCs over other pharmaceutical or biologic therapies for OA is that they have a multi-faceted therapeutic potential. They are able to persist and engraft in the joint environment to stimulate key effector cells of OA, such as macrophages, as well as chondrocytes, synoviocytes and endogenous MSCs. MSC therapeutic potential has promise to achieve what pharmacologic and other biologic OA therapies have failed to thus far: not only can MSCs modulate the inflammatory state of synoviocytes and chondrocytes [209,552], but they also release anabolic factors and undergo chondrogenic differentiation to support cartilage regeneration [575-578]. Development of feasible MSC therapies for OA has targeted the anti-inflammatory and regenerative properties with success resulting in clinical trials in both humans and horses.

MSCs can be thought of as intra-articular delivery devices to modulate inflammation during early or late OA. They have shown persistence and integration into joint tissue, however the length of time may depend on species, and the inflammatory state of the joint. For example autologous AdMSCs persisted in normal canine stifles for up to 2 weeks [579] and BMSCs in murine stifles for up to 12 weeks [580]. These models have shown that MSCs often integrate into the synovium, where they exert local paracrine effects, as outlined in the previous section [438]. Additionally, the effect of injected MSCs may persist longer due to attraction of endogenous MSCs. Attraction of endogenous MSCs has been demonstrated in murine wound healing [581], neural disease [582] and bone healing [583] models, and is dominated by the chemokine stromal cell-derived factor 1 (SDF-1) [532]. The presence of injury is important for endogenous MSC activity, as proliferation of endogenous synovial MSCs occurred with articular cartilage injury, but did not occur without injury in a murine model [584].

MSCs exert anti-inflammatory effects on synovium and cartilage via soluble mediators. Media conditioned with BMSCs exposed to TNF α and IFN γ reduced IL-1 β , MMP1 and MMP13 gene expression from synovium and increased IL-1ra from cartilage explants [585]. Manferdini, et al. [209] also found that MSCs needed inflammatory stimulation to have an anti-inflammatory effect on chondrocytes and synoviocytes. They showed that PGE $_2$ was the main soluble used by human AdMSCs to down-regulate of chondrocyte and synoviocyte production of IL-6, IL-1 β , TNF α and IL-8 [209]. As well as direct anti-inflammatory action on synoviocytes and chondrocytes, MSCs act on immune cells in the synovium, such as macrophages to have an anti-inflammatory effect. As discussed, macrophages are increased in both early and late stage OA [173,177,215], and are also a key effector cell in the synovium during OA [586]. Potential induction of an anti-inflammatory state by MSCs may be another mechanism to reduce intra-articular inflammation [4,557,558].

MSCs have the ability to differentiate *in vitro* into chondrocytes that secrete ECM and have been the main-stay of cartilage engineering that has progressed to form a product superior to fibrocartilage [587]. Autologous BMSCs produced more hyaline-like cartilage than MSCs derived from periosteum, synovium, adipose or muscle for rabbit chondral defects [588]. Therefore; BMSCs have been directly applied to osteochondral defects with success in both model species and humans [589-592]. However, some studies have found that stability of the mature chondrocyte phenotype after differentiation of BMSCs is difficult to maintain long term [593,594]. *In vitro* co-culture models have demonstrated that trophic functions of human BMSCs promote proliferation, proteoglycan and collagen production by human or bovine chondrocytes [595-597]. AdMSCs can also support chondrocyte viability during OA by reducing the expression of hypertrophic and fibrotic markers expressed by chondrocytes [598]. This suggests that trophic functions of MSCs are important for cartilage repair in addition to, or instead of, direct differentiation to chondrocytes and is supported by *in vivo* studies that have used intra-articular scaffold free injections for cartilage repair.

Current use of MSCs in human OA

Clinical trials in humans with OA are currently investigating MSCs as a therapy for cartilage regeneration and to relieve symptoms. Resurfacing of articular cartilage defects is commonly

performed with matrix-assisted autologous chondrocyte implantation (MACI). In this technique autologous chondrocytes are harvested, cultured and implanted within a matrix [599]. Using chondrocytes rather than MSCs for resurfacing articular cartilage defects requires harvest of chondrocytes from a functional part of the joint that is relatively less important for comfort during weight-bearing. When combined with high-tibial osteotomy to transfer weight to the medial condyle, MACI has improved joint function and pain for up to 5 years in young patients with medial femoral condyle OA [600], allowing joint replacement to be delayed. However, significant limitations of MACI are hypertrophy and de-differentiation of chondrocytes during culture. Therefore, BMSCs have been used in place of chondrocytes and demonstrated hyaline cartilage formation [601], long term (up to 11 years) safety, and efficacy [592]. The advantage to BMSC instead of MACI is equal pain relief with less morbidity and cost compared [602]. However, the efficacy in patients with moderate to severe OA (Kellgren-Lawrence grade $\geq 3/4$) has not been thoroughly investigated due to these patients often being excluded from cartilage resurfacing studies [602,603].

Scaffold free intra-articular injection of MSCs is simpler and takes advantage of trophic and anti-inflammatory paracrine functions of MSCs to modulate the disease environment. There are currently 18 open and 23 completed clinical trials using MSCs for OA (Table 2.1) [529]. Most clinical trials use culture expanded autologous BMSCs or AdMSCs, however 3 completed studies used allogeneic MSCs, with one that has results available (table 1). There is a range of vehicles in clinical trials for MSC delivery including hyaluronic acid, PRP and Plasmalyte-A [529]. Additionally, MSC doses range from $8\text{-}15 \times 10^7$ cells/joint. Despite differences in vehicle and dose, a systematic review on intra-articular culture-expanded MSCs demonstrated that the main adverse event related to injection was short-lived joint swelling and pain, so concluded that cultured stem cells appear to be safe [604]. The clinical trials also show significant reductions in VAS and WOMAC scores, which are two scales to assess pain and function, although it is unknown if these improvements are in-line with the current standard of care (i.e. NSAID or corticosteroid use) because none use a positive control. Modest improvements seen in radiographic and/or MRI scores [605,606] as well as histologic scores [607] could signify disease modifying effects of MSC treatment, however these improvements were not consistent in all studies.

Current use of MSCs in equine OA

The use of BMSCs to equine cartilage resurfacing of osteochondral defects has been investigated; however, the results vary because some studies fail to use an adequate control, or the treatment effect occurs over a defined time period. For example, positive results that included reduced initial PGE₂, total nucleated cell count (TNCC) and improved histological and functional quality of repair tissue were reported using AdMSCs in fibrin glue, but these were compared to no treatment controls [608]. In contrast, when compared to autologous platelet-enriched fibrin alone, addition of BMSCs did not alter biomechanical properties of cartilage at 1 year. In fact, grafts with BMSCs had increased bone edema and some horses had ectopic bone [609]. This example highlights the need for controlling for scaffold when performing cartilage resurfacing studies. Resurfacing studies have also found that treatment effects either occur early, or are delayed. For example BMSC implantation in a fibrin gel glue improved cartilage defect healing 30 days after surgery, however, there was no prolonged benefit at 8 months [468]. In comparison, scaffold-free BMSCs injected intra-articular 30 days after creating an osteochondral defect with microfracture improved tissue firmness, repair and quality at 6-12 months [469]. The success of the second approach may be due to the trophic effects of MSCs on already forming fibrocartilage.

Scaffold-free intra-articular injection of MSCs has been investigated in both naturally occurring and experimental equine OA (Table 2.2). The variable results may be an indicator of the degree of inflammatory environment that varies significantly between models, follow up time, MSC dose and source, as well as inter-observer differences in subjective outcome parameters. As discussed, MSCs respond to the inflammatory milieu by becoming “cytokine licensed”, which enables them to have an anti-inflammatory and immunomodulatory effect. The equine studies demonstrate that MSCs exposed to non-inflamed, healthy joints cause significant inflammation, evident as synovitis and increased total protein (TP), TNCC and inflammatory cytokines [455,564,573,610]. In contrast, the most severe model for intra-articular inflammation, LPS-induced synovitis, showed reduced TNCC compared to LPS used alone, demonstrating modulation of the inflammatory response to LPS [455]. Studies with variable or low intra-articular inflammation show a variable response to MSC treatment. For example, the CSU-OA model, which gave BMSC treatment 14 days after surgery did not result in appreciable levels of

reduced inflammation aside from reduction in PGE₂ [447]; and out of 165 horses with naturally occurring OA, synovitis occurred in 1.8% and there was high variability in response to treatment [611]. Two equine studies report improved lameness results; however, this was either a delayed response with no degree of improvement reported [612], or did not have a control for comparison with high variability between joints treated [611]. Given that the other studies report no improvement in lameness, further controlled studies are needed with subjective and objective lameness assessment to prove efficacy. Overall, the functional outcomes for horses (lameness) seem to be less consistent than those observed in the human clinical trials, which may be due to increased pain and OA progression in human compared to equine patients and the high standard of pain relief needed for horses to return to previous level of use. It is clear that MSC treatment for horses could be optimized to improve functional and pathobiologic outcomes in horses with joint disease.

Table 2.1 - Published clinical trials using scaffold free intra-articular MSCs for the treatment of OA in humans

Indication/ joint	OA Grade	MSC source	Dose	Vehicle	Control	Outcome	Ref.
Knee OA	Moderate-severe	Auto-BMSC	8-9x10 ⁶	N/A	None	Pain improved 24-50% compared to pre-injection after 6 months	[613]
Knee, ankle or hip OA	KL IV	Auto-BMSC	20-24x10 ⁶	Physiological serum	None	Improved pain and function at 30 months – Pilot study	[614]
Knee OA	KL II-IV	Auto-BMSC	40x10 ⁶	Lactated Ringers	None	Significantly improved functional and pain scores at 1 year	[615]
Knee OA	KL II-IV	Auto-BMSC	40x10 ⁶	Lactated Ringers	None	Continuation of [615]. No significant reduction in pain improvement from 1 year to 2 years. Improved cartilage scores on MRI compared to 1 year.	[616]
Knee OA	KL ≥ 2	Auto-AdMSC	Low: 10x10 ⁶ Med: 50x10 ⁶ High: 100x10 ⁶	Saline	None	Pain and function improved significantly in high dose group only at 6 months, trend for improvement in other groups. No significant change in radiographic scores. Size but not depth of cartilage lesion significantly decreased in high dose and had improved biopsy histologic score.	[607]
Knee OA	KL II-IV	Allo-BMSC	40x10 ⁶	N/A	HA	Significantly decreased pain and function scores in MSC group	[617]
Knee, ankle or hip OA	KL III-IV	Auto-BMSC	5x10 ⁵ cells/kg/bw	Saline	None	Improved pain and function at 6 and 12 months, not at 30 months. Reduced bone edema on MRI up to 6 months	[606]
Knee OA	KL ≥ 2	Auto-BMSC	Dose 1: 10x10 ⁶ Dose 2: 100x10 ⁶	HA	HA	Pain and function scores improved significantly at 12 months in MSC groups compared to control. Radiographic and MRI improvements seen in high dose group.	[605]
Knee OA	KL 2-3	Allo-BMSC (pooled)	25x10 ⁶ 50x10 ⁶ 75x10 ⁶ 150x10 ⁶	Plasmalyte+2mL HA Pre-medication with IA hydrocortisone	Placebo (P-lyte)+2mL HA Pre-medication with IA hydrocortisone	Injection site swelling and joint pain in some 75M and 150M patients. 25M group had maximum pain and function improvement at 12 months. 150M group did not have improved function/pain. No change in MRI	[618]

Table 2.2 - Published pre-clinical and clinical trials of scaffold free intra-articular MSCs for the treatment of OA in horses

Joint	Model	MSC source	Dose	Vehicle	Control	Outcome	Ref
Middle carpal	CSU osteochondral fragment	Auto-BMSC	5.6-15x10 ⁶	Saline	Saline	Treatment did not affect lameness, flexion test, radiographs or cartilage/synovial gross exam/histology compared to control. Decrease in PGE ₂ with BMSC treatment.	[447]
Radiocarpal	Healthy joints	Auto- and Allo-umbilical cord blood and tissue MSCs	7.5x10 ⁶	Saline	Saline tibiotarsal joint	Joint effusion, no difference in lameness between groups, increase in synovial TP and TNCC with MSC treatment for 24 hours, no difference between MSC groups.	[610]
Medial femorotibial	Osteochondral defect	Auto-BMSC 1 months after defect creation	20x10 ⁶	HA	HA	No difference between MSC and control for lameness, flexion, radiographs, MRI, histology of repair tissue. MSC joints had firmer cartilage repair after 6 months, no reduction in firmness 6-12 months, increased area of repair tissue	[469]
Bone-spavin (Tarso-metatarsal)	Naturally occurring OA	Auto-AdMSC	5x10 ⁶	Saline	Betamethasone No treatment	No change in lameness at 30 days but reduced at 60 days. 180 days improvement remained in MSC group but not betamethasone group. Decreased neutrophil count at 90 days in MSC and betamethasone compared to pre-injection.	[612]
Metacarpo- and metatarso-phalangeal	Healthy joints	Allo- Auto-, Genetically modified Auto-, Xeno(human) BMSC	Not stated	Not stated	None	60 days after injection increased synovial cellularity compared to non-injected reference, TNCC significantly increased in xeno group. <i>In vitro</i> re-exposure caused increased immunogenicity in xeno-	[619]
Metacarpo- and metatarso-phalangeal	Healthy joints	Allo- Auto-, Genetically modified Auto-, Xeno(human) BMSC	15x10 ⁶	Gey's balanced salt solution	Gey's balanced salt solution	All increased TNCC, TP, IL-6 compared to control. No difference between Auto- and Auto-GM MSCs but increase for allo- and xeno- compared to Auto- for TNCC	[573]
Stifle, fetlock, pastern, coffin	Naturally occurring OA	Allo- peripheral blood MSCs Native or chondrogenic induction	Not stated	PRP	None	1.8% (of 165 horses) synovitis in first week, improved return to work at 18 weeks compared to 6 weeks, chondrogenic MSCs resulted in higher return to work in distal limb joints but not stifle joints	[611]
Table continues on next page							

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Joint	Model	MSC source	Dose	Vehicle	Control	Outcome	Ref.
Stifle	Meniscal, ligament, cartilage injury	Auto-BMSC + arthroscopy	15-20x10 ⁶	Autologous serum/5% DMSO +HA	None – results compared to previous literature	Unilateral affected horses 45% return to previous work, 23% return to work, 32% failure to return to work. In comparison to previous studies without MSCs, more meniscal injuries returned to work/ previous level of work. 3/33 horses had acute joint inflammation after MSC injection	[277]
Radiocarpal and tibiotarsal	Healthy joints	Radiocarpal: Allo-BMSCs (pooled) Tibiotarsal: Auto-BMSC	Repeat doses x 3 *	LRS	LRS	Mild synovitis in all treatment groups that resolved spontaneously by day 10. Significantly higher TP and WBC in all treatment groups. * 1: 25x10 ⁶ Auto, 2: 25x10 ⁶ Allo, 3: 25x10 ⁶ Allo repeat	[564]
1. Tibiotarsal - healthy 2. Middle carpal - LPS	1. Healthy joints 2. LPS synovitis	Allo-cord blood MSCs	1. 30x10 ⁶ 2. 10x10 ⁶	1. Saline 2. Saline + 0.5ng LPS	1. Saline 2. Saline + 0.5ng LPS	Increased lameness (2-4 AAEP) for 48 hours, TP, TNCC, PGE ₂ and biomarkers with MSCs in healthy joints. Increased lameness, joint circumference, TP in LPS/MSC limbs compared to LPS control, but decreased TNCC.	[455]

Spheroid culture of mesenchymal stem cells to enhance therapeutic potential

Pre-conditioning of MSCs to alter therapeutic potential

Disease environments vary in the degree of inflammation, oxidative stress, ECM degradation, ischemia and immunologic response, which not only influences the activity of delivered MSCs, but can also reduce their survival or retention rate at the site of injury [496]. Compared to the *in vivo* environment, MSCs are cultured in conditions with higher oxygen, nutrients and lack of an extracellular matrix. However, pre-conditioning by exposure of MSCs to an environment similar to the disease environment *ex vivo* allows improved survival of hypoxic MSCs in ischemic heart disease [620-622], heat shocked MSCs in conditions of oxidative stress [623] and the immunomodulatory activity of IFN γ treated MSCs in murine graft-vs-host disease [549].

Current methods of pre-conditioning used in pre-clinical trials include hypoxia during MSC culture, exposure to exogenous cytokines and/or three-dimensional culture. *In vitro* hypoxia increases indolamine 2,3 deoxygenase (IDO), which is a factor human MSCs use to down-regulate T cell proliferation and induce T regulatory cells that modulate immune response [624,625]. *In vivo* hypoxic treated murine allogeneic BMSCs have an enhanced ability to engraft due to reducing inflammatory cell infiltration compared to normoxic BMSCs [626]. As stated previously, exposure of MSCs to IFN γ , TNF α , IL-1 and/or IL-17 activates them into a “cytokine licensed state” that produces increased immunomodulatory secretory factors [627-633]. Molecules that activate the innate immune system, such as LPS, also induce a cytokine licensed state and increase growth-factor release [634].

Three-dimensional culture of MSCs as spheroids is a form of pre-conditioning that improves anti-inflammatory properties in models of murine peritonitis [3] and of macrophage behavior *in vitro* [4]. BMSCs naturally form spheroids *in vivo* [2] and in doing so, increase anti-inflammatory activity through cell-cell and cell-matrix interactions that up-regulate paracrine pathways for the modulatory factors TSG-6, IL-6 and PGE $_2$ [2,5], as well as creating a hypoxic environment at the spheroid center [635]. Furthermore, there are significant advantages of spheroid culture over hypoxic or exogenous cytokine pre-conditioning methods because spheroid culture does not require a specialized incubator, or risk of breaking hypoxemic conditions when

flasks are removed for assessment and there is no risk of inadvertently delivering trace amounts of an exogenous cytokine as with recombinant cytokine preconditioning.

Mechanisms and methods of spheroid MSC culture

Murine and human MSCs from bone marrow, adipose tissue and umbilical cord have the ability to spontaneously form three-dimensional aggregates when there is no adherent surface [3,4,635-637]. Although the methods of MSC self-aggregation are incompletely understood, it is likely that close proximity of cells causes cell-cell aggregation through cell adhesion molecules and, as extracellular matrix (ECM) is produced, further adhesion through cell-ECM interactions.

BMSCs have a high expression of the cell adhesion molecules N-cadherin and cadherin-11, other MSC types have different expression of cell adhesion molecules [638,639]. Initial cadherin-cadherin binding to form BMSC spheroids is exemplified by disruption of spheroid formation in the presence of EDTA, which binds calcium to inhibit cadherin binding [640]. Cell-ECM interaction likely increases overtime and is involved in stabilization of the spheroid, this is shown by an increase in BMSC spheroid stiffness over time [641], which plays a role in signal transduction [642-644]. As spheroids of MSCs organize, cells become highly organized in space, with a rounded morphology centrally and flattened morphology at the outer edges [635]. This organization also comes with a decrease in spheroid size, it has been reported that human BMSC spheroids reduce in size by ~300µm during aggregate compaction [3,5,645].

Morphological alterations also occur on a cellular level, in its simplest form this was seen as spheroid derived human BMSC cells being about 1/3rd the size of monolayer cells [3,5,646]. Cytoskeletal changes responsible for this size decrease include contraction of actin, one of the major components of the cellular cytoskeleton, and is integral at regulating fusion, compaction, and functional activation of BMSC spheroids as a whole [5].

A number of different culture methods using spontaneous or forced aggregation of MSCs have been used to form scaffold-free spheroids from MSCs. Johannes Holtfreter first described scaffold-free spheroid culture of mammalian embryonic cells in 1944 using a hanging drop technique that relies on surface tension in suspended drops of media [647]. He described spontaneous aggregation of cells into a “radially symmetrical structure” when suspended in media hanging on an agar plate lid. Since that time hanging drop culture techniques have been

modified to increase efficiency, spheroid uniformity and allow high-throughput applications. Modified techniques range from using inverted micro-well plates [648], to specialized hanging drop plates [649]. Spontaneous aggregation can also be achieved through culture on non-adherent surfaces. The surface type can alter both the size and uniformity of spheroids, for example, on polydimethylsiloxane (PDMS) embryonic stem cells formed uniform spheroids that had >2-fold increase in spheroids 100-300 μ m compared to 3 other manually produced and 2 commercially available low adherent surfaces [650]. However, cell adhesion and viability can be affected by variability in surfaces that are produced manually such as PDMS [651]. The main limitations of non-adherent surfaces are variations in spheroid size and viability compared to hanging drop [645]. Different surface treatments from chitosan-hyaluron membranes to poly(ethylene glycol) micro-patterned plates have been investigated to promote homogeneity of MSC spheroids [652-654], and physical separation by culture in 96 well non-adherent plates has been used [645,655]. Forced aggregation via centrifugation of human [656] and murine [636] BMSCs has been used to form spheroids. After initial aggregation the cell aggregates were maintained in a rotating flask for long-term culture. The advantage of this technique is it allows fast formation of spheroids and is a high-throughput technique. However, direct comparison of forced aggregation and spontaneous formation techniques on the functional characteristics of spheroids has not been performed, and most functional studies have used spontaneous aggregation.

Characteristics of spheroids that enhance therapeutic potential

MSC spheroids have shown anti-fibrotic properties [657], resistance to ischemic conditions [658,659], increased homing capacity [660], anti-inflammatory properties [3,4] and enhanced differentiation capabilities [636]. Initial spheroid culture of human BMSCs showed alteration of 710 genes compared to traditional monolayer culture, up-regulating genes responsible for developmental processes, antioxidant activity, response to stimuli and signal transduction [635]. Subsequent targeted research found that spheroid-induced increases in PGE₂, TSG-6 and IL-6 expression that peaked at 72-96 hours of aggregation were mainly responsible for enhanced anti-inflammatory and immunomodulatory properties [2-5]. PGE₂, TSG-6 and IL-6 production is due to self-activation of IL-1 and Notch signaling [2], and is caspase-dependent [2,5]. The involvement of these signaling pathways, that are usually up-regulated in inflammatory

environments, validates the “cytokine licensed” effect that spheroid formation has, without the need for exposure to exogenous inflammatory mediators. As well as self-activation of a cytokine licensed phenotype, BMSC spheroids also up-regulate the SDF-1 receptor, CXCR4, which is involved in stem cell homing and is down-regulated in traditional monolayer culture [660]. SDF-1 (stromal cell-derived factor 1/ CXCL12) is a chemokine that promotes angiogenesis and endogenous stem cell recruitment [661,662], it is increased in spheroids from human gingival derived [663] bone marrow derived [5] MSCs, so spheroids could promote endogenous stem cell attraction *in vivo*.

Spheroids have increased anti-inflammatory activity than traditional monolayer cultured cells, demonstrated *in vitro* and *in vivo*. Interest in the anti-inflammatory activity of MSC aggregates was due to the observation that intravenously infused human BMSCs were trapped in the lung as micro-emboli and had increased expression of TSG-6 after 12-24 hours of aggregation [3].

TSG-6 (TNF α stimulated gene/protein 6) is a small glycoprotein that is induced in the presence of TNF α and IL-1 β and counteracts their inflammatory effects [664]. By local paracrine effects, the aggregates were able to limit inflammatory mediated deterioration of cardiac tissue.

Subsequent *in vitro* culture of human BMSC spheroids found that TSG-6 levels were 1000-2500 times higher than traditional monolayer cultured BMSCs, and were able to decrease TNF α production from LPS stimulated murine macrophages [3]. The only *in vivo* anti-inflammatory spheroid study performed using a murine model of zymosan-induced peritonitis showed reduced peritoneal protein, pro-inflammatory cytokines TNF α , IL-1 β and PGE₂ compared to control animals or those treated with monolayer BMSCs [3]. The key paracrine mediator released from human BMSC spheroids is PGE₂ and causes phenotypic alteration of macrophages in a dose-dependent manner from a high TNF α production (M1) phenotype to a low TNF α , high IL-10 production (M2) phenotype [4]. Additional pre-conditioning of human BMSC forced-aggregation spheroids by exposure to IFN γ and/or TNF α further enhances their secretion of immunomodulatory cytokines and macrophage modulation [665]. Although the overall anti-inflammatory mechanisms of spheroids are incompletely understood, they likely involve both direct anti-inflammatory mediators, such as TSG-6, as well as immunomodulatory mediators, like PGE₂ and IL-6 to cause an anti-inflammatory phenotype of immune cells.

Summary

This section has highlighted the ability of spheroid culture to modulate the anti-inflammatory properties of MSCs. The spheroid culture technique has been used for BMSCs in both mice and humans, however, there are no published reports on the ability of equine BMSCs to form spheroids, or if their anti-inflammatory potential is similar. As discussed in chapter 1, inflammatory processes characterize the initiation and propagation of OA. There is also infiltration and activation of immune cells in the synovium; in particular, synovial activated macrophages are increased in both early and late stage OA [173,177,215,586]. MSCs cultured in traditional monolayer culture have already shown the ability to modulate inflammation of synoviocytes and chondrocytes [585,666], but it is unknown if spheroid cultured MSCs will have enhanced anti-inflammatory potential or if this will translate to clinical efficacy *in vivo*. The horse is a good model of human OA [440] with established, predictable, models of both acute joint inflammation [253], and chronic [27] OA. Therefore, investigation of equine BMSC spheroid culture and application *in vivo* is warranted to investigate the anti-inflammatory potential of spheroid cultured BMSCs for joint inflammation.

CHAPTER 3

Three-dimensional Culture Enhances Anti-Inflammatory Potential of Equine Mesenchymal Stem Cells Regardless of Inflammatory Stimulation

Abstract

Three-dimensional spheroid culture of human bone-marrow derived mesenchymal stem cells (BMSCs) increases their anti-inflammatory activities *in vitro* and *in vivo* via direct production of anti-inflammatory mediators, as well as modulation of immune cells. The horse is affected by both musculoskeletal and systemic inflammatory diseases and has been used as a large animal model for human disease, specifically osteoarthritis (OA). However, it is unknown if spheroid MSCs, in general, can be used to benefit OA, if spheroid culture of equine BMSCs (eBMSCs) is possible, or if they have a similar phenotype to human spheroids. Therefore, the purpose was to validate the culture of eBMSCs as spheroids and to assess the effect on gene expression and production of inflammatory and anti-inflammatory mediators under LPS stimulation *in vitro*. We investigated differences in spheroid and monolayer eBMSC cultures over 120 hours of propagation and over 24 hours of lipopolysaccharide (LPS) stimulation. There were no differences in cell viability and minimal alterations in stem cell markers of stemness over 120 hours of culture, with all cultures maintaining $\geq 99\%$ viability at 120 hours. Spheroid culture significantly altered the secretion and gene expression of the immunomodulatory markers TSG-6, SDF-1, PGE₂ and IL-6 independent of LPS stimulation, but did not produce measurable levels of the inflammatory cytokines TNF α or INF γ . There was cell line dependent variation in IL-10 production. Overall the alterations are similar to those published for human BMSC spheroid cultures with enhanced anti-inflammatory activities so further investigation of eBMSC spheroid use in horses is warranted.

Introduction

Mesenchymal stem cells (MSCs) have the ability to alter the inflammatory behavior of immune cells causing anti-inflammatory and immunomodulatory outcomes. Paracrine production of

soluble factors such as PGE₂, IL-10 and TSG-6 have been found to be responsible for inducing macrophages to an anti-inflammatory phenotype, modulating the proliferation of T cells and antigen presentation of dendritic cells [554,667], and reducing neutrophil burden [562]. Specific paracrine mediators used by MSCs can depend on species type, however, PGE₂ has been found to be the main mediator used by murine, human and equine MSCs derived from bone (BMSCs) [553,668,669]. Additionally, the anti-inflammatory protein TSG-6, stimulated by the presence of TNF α or IL-1, has been suggested as a biomarker to predict the efficacy of human BMSCs at attenuating sterile inflammation *in vivo* [670].

The anti-inflammatory and immunomodulatory potential of MSCs is largely dependent on the disease environment. A concept of “cytokine licensing” whereby MSCs are activated into an anti-inflammatory and immunomodulatory state in the presence of inflammatory mediators such as IL-1, TNF α , LPS or IL-17 was proposed [671]. The use of exogenous inflammatory mediators during BMSC culture has been used to enhance the anti-inflammatory effects of MSCs, and has most recently been used for therapeutic applications such as radiation induced injury [672]. Another way to enhance anti-inflammatory potential of BMSCs is three-dimensional culture as spheroids. Spheroid culture of human BMSCs alters the production of 710 genes involved in antioxidant activity, response to stimuli and signal transducer activity [635]. Spheroid cultured cells have the ability to reduce PBMC proliferation, modulate systemic inflammation in murine models [3] and influence macrophages to change to an anti-inflammatory phenotype that produces enhanced IL-10 and reduced TNF α [4,646]. These studies demonstrated that an anti-inflammatory phenotype in spheroids is characterized by enhanced production of PGE₂ and TSG-6, and the mechanism for this has since been found to involve IL-1 signaling and characteristically increase PGE₂ and IL-6 [2,5]. Alterations of SDF-1/CXCL12, a chemotactic factor, have also been found in MSC aggregates [660].

Horses represent both a model species in which to study inflammatory orthopedic and immunomodulatory disease, as well as patients afflicted by both orthopedic and systemic inflammatory diseases. Horses are primarily used for athletic purposes, and represent a model of naturally occurring osteoarthritis due to a prevalence of 83.5% in horses ≥ 15 years old, and up to a third of 2- to 3-year-old Thoroughbred racehorses [17,25]. Equine models of uveitis [673] and dermatitis [674] represent models of other inflammatory or immune mediated diseases. eBMSCs

have been investigated and are currently in clinical use for tendinitis [464], intra-articular soft tissue injury [277] and cartilage resurfacing [469]. Although eBMSCs have been found to have anti-inflammatory effects *in vitro* including suppression of lymphocyte proliferation [675] and produce anti-inflammatory cytokines under inflammatory stimulation [676], monolayer cultured eBMSCs could not significantly alter the progression or inflammatory mediators in *in vivo* models of equine osteoarthritis [447]. An enhanced anti-inflammatory stem cell treatment is warranted, given the high prevalence of equine osteoarthritis, and negative effects of currently used corticosteroids on equine cartilage health [296,316,330]. It is unknown if eBMSCs will be able to spontaneously form spheroids, or if they will have similar characteristics to human spheroid cultured BMSCs therefore making horses a good model for studying therapeutic applications of spheroid cultured BMSCs.

The purpose of this study was to validate the culture of eBMSCs as spheroids and to assess the effect on gene expression and production of inflammatory and anti-inflammatory mediators under LPS stimulation *in vitro*. We hypothesized that 120 hours of spheroid culture would not affect the viability or stem cell marker expression of eBMSCs and that the cell biomass would increase over time. We also hypothesized that spheroid culture would alter the inflammatory and anti-inflammatory mediators produced compared to monolayer culture, and respond differently to LPS exposure.

Materials and methods

eBMSC Collection and Characterization: Three eBMSC cell lines were isolated from 3 horses with IACUC approval. eBMSCs were isolated and expanded in low-glucose GlutaMax Dulbecco's modified eagle medium (GlutaMax DMEM, Invitrogen) with 110µg/mL sodium pyruvate (Gibco) plus 10% MSC FBS (Gibco) and 100U/mL sodium penicillin, 100µg/mL streptomycin sulfate (Sigma) at 37°C, 5% CO₂, and 90% humidity and passaged at 70-80% confluence. They were determined to undergo tri-lineage differentiation, and to be Oct-4 and CD90 high and MHCII negative by flow cytometry. First passage eBMSCs were cryopreserved in liquid nitrogen vapor until use.

eBMSC cell culture: eBMSCs were recovered from cryopreservation, expanded as monolayer cultures then were passaged at 70%-80% confluence for subsequent culture as monolayer and

three-dimensional spheroids. All eBMSCs were grown in media comprised of: low-glucose GlutaMax DMEM with 110µg/mL sodium pyruvate (Gibco) plus 10% MSC FBS (Gibco) and 100U/mL sodium penicillin, 100µg/mL streptomycin sulfate (Sigma), and incubated at 37°C, 5% CO₂, and 90% humidity with media supplementation every 48 hours.

Part 1: Validation of spheroid culture

eBMSC monolayer cell culture: 50,000 passage 3 eBMSCs were cultured as a monolayer in each well of a 6 well adherent tissue culture plate for 5 days. Culture medium was exchanged every 48 hours.

eBMSC spheroid cell culture: 50,000 passage 3 eBMSC spheroids were formed using a hanging drop technique in Biopreacta™ 96-hanging drop plates. A spheroid size of 50,000 was found to be optimum for viability to 14 days of culture in a pilot study. Each spheroid was harvested into a well of a 96-well non-adherent tissue culture plate once formed at 72 hours. Culture medium was exchanged every 48 hours as per manufacturer instructions.

Micrographs: Micrographs of randomly chosen spheroids and monolayer cultured cells were obtained in triplicate using an EVOS XL digital inverted microscope on 20x20 objective at 24, 48, 72, 96 and 120 hours.

DNA quantification: At 0, 24, 48, 72, 96 and 120 hours, individual spheroids were harvested and individual wells of monolayer-cultured cells were trypsinized in quadruplicate and placed in 500ul microcentrifuge tubes. The samples were centrifuged at 10,000 RPM for 10 minutes and the supernatant was removed. Samples were stored at -80 degrees Celsius until DNA quantification. Upon thawing, the cells were lysed in 100ul of distilled sterile water using a freeze-thaw technique. NanoDrop 2000c spectrophotometry was used to assess DNA quantity, and validated with Quant-iT™ dsDNA Broad-Range Assay Kit (Invitrogen™).

Assessment of viability: At 0 and 120 hours monolayer cultured cells were trypsinized and spheroids were harvested. Cells were centrifuged at 800g for 10 minutes at 4°C and supernatants were removed. For quantitative viability assessment, 20 spheroids were dissociated into a single cell suspension using 400ul of Liberase TL (Roche) working solution that was at a final collagenase activity of 1.2 Wunsch units/ml [637]. Following washing in PBS and suspension of

eBMSCs in 50ul (dissociated spheroid cells) or 100ul (monolayer cells) of PBS, cell viability ratios were measured by Live/Dead assay (Invitrogen, UK). 1 ml of PBS containing $2.5 \mu\text{l ml}^{-1}$ of $4 \mu\text{M}$ ethidium homodimer-1 (EthD-1) assay solution and $1 \mu\text{l ml}^{-1}$ of $2 \mu\text{M}$ calcein AM assay solution was prepared. 50 μl or 100 μl of the Live/Dead solution was added to each sample of spheroid or monolayer cells, respectively, and the samples were incubated for 20 minutes in darkness at room temperature. The samples were transferred to a hemocytometer then viewed using EVOS XL digital inverted microscope with 494 nm (green, calcein) and 528 nm (red, EthD-1) excitation filters. Images were captured using integrated software. For quantitative analysis Live/Dead cell counts were taken from 10 primary hemocytometer squares. A preliminary trial found no statistical difference between cell counts taken using the Live/Dead assay or Trypan Blue for monolayers or spheroids ($p = 0.305$ and $p = 0.248$, respectively). The viable and non-viable cells counts were recorded in Microsoft Excel and converted as a percentage of live cells/dead cells for analysis. For viability assessment in situ, media was removed from wells with spheroids or monolayer cultured MSCs in triplicate. PBS and the prepared Live/Dead assay solution was added 1:1 to cover the surface of the wells (50ul:50ul for spheroids in 96 well plate, 200ul:200ul to cover monolayers in 6 well plates). The cells were incubated, fluorescence was assessed and images captured in situ as above.

Expression of stem cell surface markers: At 0 and 120 hours of culture, spheroids and monolayer cultured cells in quadruplicate were lysed with Trizol reagent and stored at -80°C until RNA isolation. Quantitative RT-PCR (qPCR) utilizing pre-designed (CD44, CD90) or custom (CD105, Oct-4, GNL3) TaqMan primers and MGB probes were used to assess expression of stem cell markers. RNA was isolated using Trizol, and purified using a commercially available column-based protocol (RNeasy, Qiagen) and included an on-column DNase treatment to exclude genomic template contamination. First strand complimentary DNA (cDNA) was synthesized by use of oligo(dT) priming and a commercial reverse transcriptase kit; non reverse transcriptase controls were produced (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA). Complimentary DNA was amplified by fluorescent real time PCR (Absolute Quantitative PCR: ABI PRISM 7300 Sequence Detection System, Applied Biosystems, Foster City, CA) using custom or pre-designed primers and MGB probes. For custom sets, sequences were obtained from published literature, designed to span exon-exon

boundaries using available sequence data on GenBank (Primer Express Software Version 3.0, Applied Biosystems, Foster City, CA) and were cross-referenced against NIH Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/>). Relative gene expression was calculated using the comparative threshold cycle method ($\Delta\Delta C_t$) using GAPDH RNA expression as an endogenous control, after verifying gene expression stability in monolayer and spheroid eBMSC samples compared to other potential endogenous controls (B2M, RPL13A, RPL32, HPRT1) using GeNorm algorithm normalization.

Table 3.1 - Applied Biosystems Pre-designed Primer-Probe Sets

Gene	Product ID
CD-90	Ec03470737_m1
IL-10	Ec03468646_m1
IL-6	Ec03468679_m1
IFN γ	Ec03468604_m1
TNF α	Ec03467869_m1
TSG-6	Ec03468339_m1
CD44	Ec03468870_m1

Table 3.2 - Custom Primer-probe sequences:

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')
Oct-4	CAGCTCGGGCTCGAGAAG	TTCTGGCGACGGTTGCA	ACGTGGTACGAGTGTGG-6FAM
CD105	TCCACATCCTCTTCCTGGAGTT	GGACCTTTGGATAGTCAGCTTCA	CCAAGGGATGTGTCAGAG-6FAM
CXCL12/SD F-1	GCCAGAGCCAACATCAAA CA	CACGATCTGAAGGGCACAG TT	CTTAAAATCCTCAACAC TCC-6FAM
mPGES-1	AGGGTGTGTTGCGATTTCTTTT	GGGCACCGAGCATAATGC	CGGCCGGAGTTCA-6FAM
GNL3	TTCGGAAGCTGAGCTAAGG	CTGTCAAGCTTCTGCTGCTGTT	AACAGCGGCTTGAAG-6FAM
GAPDH	CAAGTTCCATGG CACAGTCAAG	GGCCTTTCCGTT GATGACAA	CCGAGCACGGGAAG-6FAM

Part 2: Anti-inflammatory characterization

eBMSC monolayer cell culture: 100,000 passage 3 eBMSCs were plated in each well of a 24 well adherent tissue culture plate and allowed to settle for 48 hours.

eBMSC spheroid cell culture: 50,000 passage 3 eBMSC spheroids were cultured using the Bioperfecta™ hanging drop technique previously described with media supplementation at 48

hours of culture. Two spheroids were harvested into each well of a 24-well non-adherent tissue culture plate at 72 hours of culture.

LPS stimulation: Existing media was exchanged with 1ml of low protein media comprised of low-glucose GlutaMax DMEM supplemented with 5% MSC qualified FBS and were exposed to either 0ng/ml (control) or 100ng/ml (exposed) LPS (O55:B55 Sigma™), in triplicate. At 0, 6, 12 and 24 hours, media was collected in 200µL aliquots and whole cells were treated with Trizol reagent or harvested by trypsinization (monolayer) or centrifugation (spheroids). All samples were cryopreserved at -80°C until analysis.

DNA quantification: At the time of LPS stimulation and after 24 hours of incubation with 0ng/mL or 100ng/mL, monolayer MSCs were trypsinized and spheroids were harvested in triplicate. All cells were centrifuged at 800g for 10 minutes; the supernatant was removed and cell pellets were cryopreserved at -80°C. DNA quantification was performed using NanoDrop 2000c spectrophotometry as described previously.

Assessment of viability: At the time of LPS stimulation and after 24 hours of incubation with 0ng/mL or 100ng/mL LPS, wells of monolayer MSCs were trypsinized and spheroids were harvested in quadruplicate. All cells were centrifuged 800g for 10 minutes at 4°C and washed in PBS. Assessment of viability was performed using a Live/Dead assay (Invitrogen, UK) on pooled spheroids (n = 8 spheroids) dissociated with Liberase (Roche) and pooled trypsinized monolayer cells (n = 4 wells), as well as triplicate samples in situ, as described previously.

ELISA: Samples of media for ELISA were immediately cryopreserved at -80°C as 200µL aliquots until analysis. ELISA was used to determine the concentrations of TNF-α, IL-6, IL-10, PGE₂, IFN-γ and SDF-1 using commercially available kits (TNF-α, Endogen, Inc.; PGE₂, Cayman Chemical Inc.) or primary reagents validated for use in the horse (IL-10, SIGMA™; IL-6, USCN Life Sci.; R and D Systems Duo-Set™, IFN-γ; R and D Systems Duo-Set™; SDF-1, R and D Systems) to manufacturer recommendations. ELISA data was normalized to DNA quantity taken as previously described to enable comparison of pg/ngDNA.

Gene expression analysis: The techniques described previously for expression of stem cell surface markers were used for RNA isolation, cDNA production and target amplification

analysis using custom (SDF-1, mPGES-1) and pre-designed (IL-10, IL-6, IFN γ , TNF α , TSG-6) primers and MGB probes, with GAPDH as the endogenous control.

Statistical Evaluation:

A power of 0.80 was predicted with 3 replicate cell lines based on preliminary data when eBMSCs were exposed to LPS or cultured as spheroids (G*Power 3.1, Dusseldorf). Paired t tests were performed between spheroid and monolayer culture on viability data at 120 hours of culture and on all treatment groups before and after LPS exposure. Change in spheroid or monolayer DNA quantity over 120 hours was analyzed by regression analysis. qPCR data was normalized to the endogenous control and fold increase calculated as a ratio compared with control no LPS monolayer gene expression. Normal probability plots showed a lognormal distribution of qPCR data, so data was log transformed prior to analysis. Stem cell marker gene expression was compared between spheroid and monolayer cultures at 120 hours using paired t tests. A three-way analysis of variance was performed to investigate overall effects of LPS stimulation (LPS and no-LPS), culture condition (spheroid and monolayer), and time for qPCR and ELISA protein production data. In the absence of a significant time effect, the effects of culture condition and LPS stimulation were assessed with a two-way analysis within each time point (0, 6, 12 and 24h). Tukey HSD pairwise comparisons were used to assess specific relationships if the culture condition or LPS stimulation were significant effects. Data was recorded in Microsoft Excel and statistical analyses were performed with SAS JMP® Pro 11.0.0. A priori significance was set at $p \leq 0.05$. Data is presented as mean $\pm$ S.E.

Results

Part 1: Validation:

eBMSCs aggregated into spheroids in hanging drop culture and had gained spherical shape by 72 hours (Fig. 3.1). Cell viability of monolayer and spheroid culture was unchanged at 120 hours ($p = 0.2744$); viability for both culture groups was $\geq 99\%$ at 120 hours. DNA quantity in monolayer culture increased over time ($p < 0.0001$, Fig. 3.2), but decreased in spheroid culture ($p = 0.0007$, Fig. 3.2). From 24 hours, monolayer cultures had higher DNA quantity than spheroid cultures, but by 120 hours this difference was not significant (Fig. 3.2).

The stem cell marker GNL3 was highly expressed in spheroid culture compared to monolayer culture at 120 hours ($p = 0.0481$, Fig. 3.3), with a trend for an increase in CD90 ($p = 0.0653$). There were no significant differences in the expression of CD44, CD105 or Oct4 at 120 hours of culture.

These results suggest that the spheroid culture does not decrease MSC viability, and has minimal effect on stem cell markers. GNL3, is associated with cell cycle regulation and may be linked to the decreased DNA quantity of spheroids over time, suggesting reduced cell proliferation compared to monolayer culture.

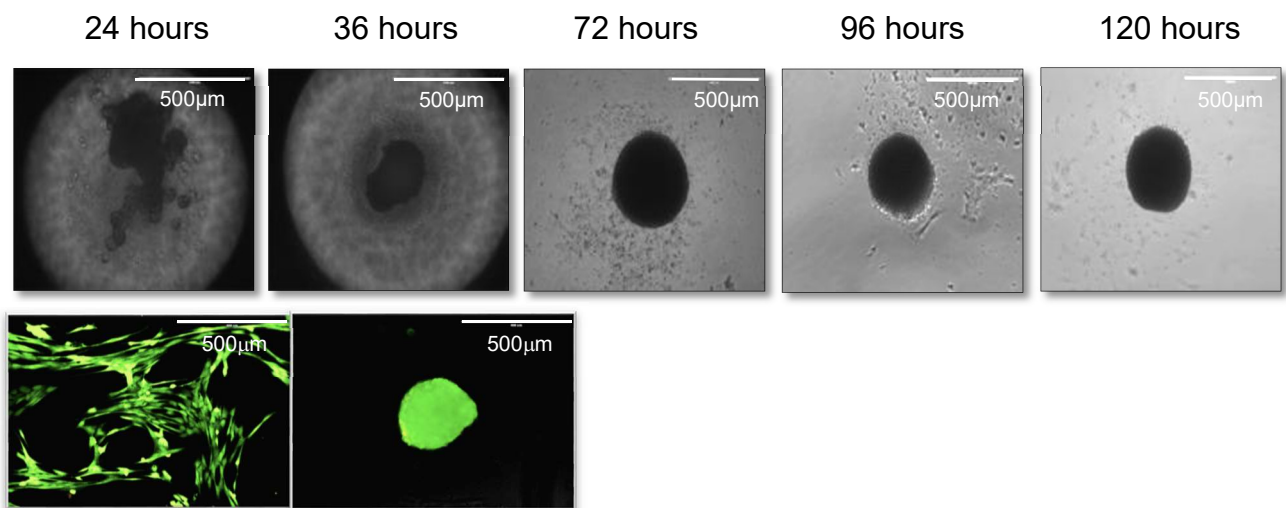


Figure 3.1 –Spheroid formation

Top: Micrographs (4x) of eBMSCs aggregating to form spheroids over 120 hours.

Bottom: Example of in situ live-dead staining of monolayer (left) and spheroid (right) eBMSCs at 120 hours.

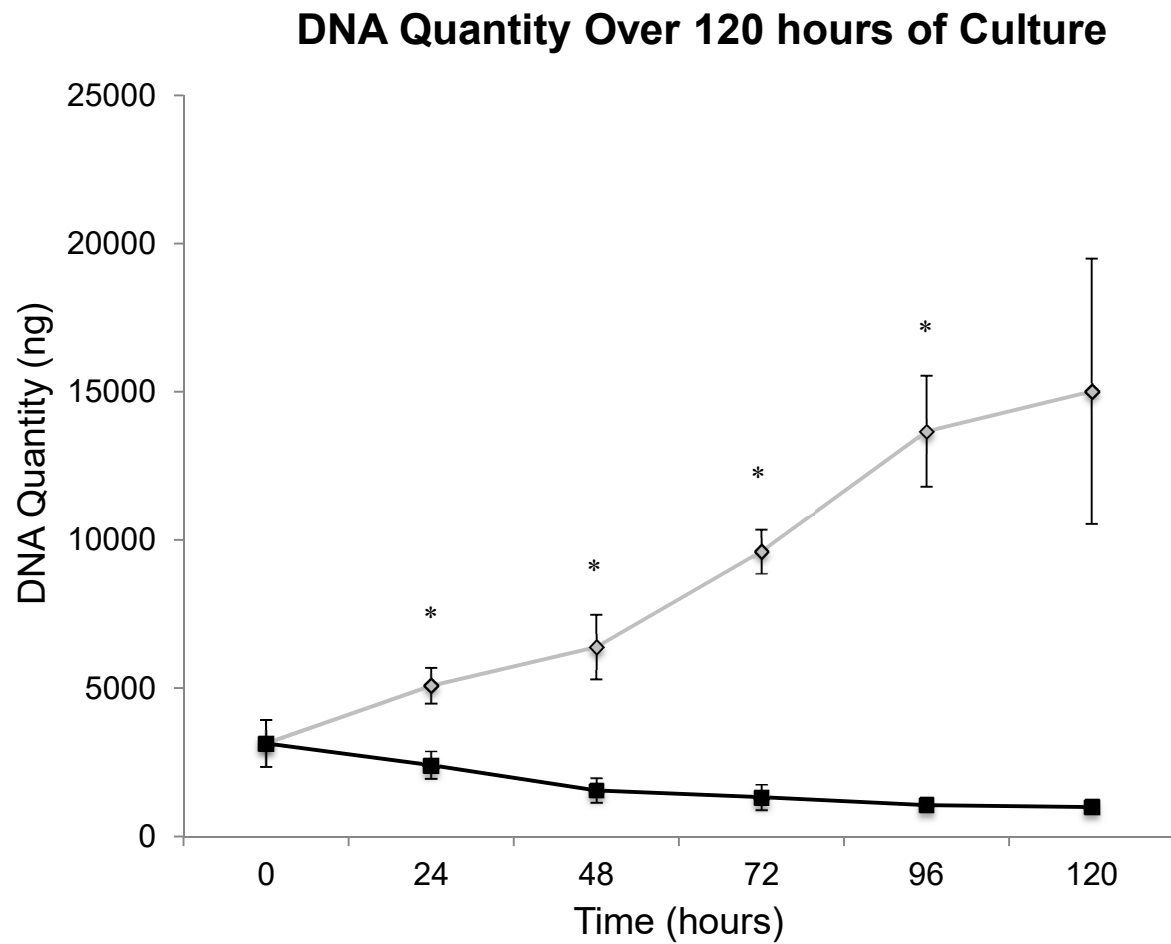


Figure 3.2 - DNA quantity

DNA (ng) over time in monolayer (◆) and spheroid (■) cultures. Overtime there was an increase in monolayer DNA quantity ($p < 0.0001$) but decreased in spheroids ($p = 0.0007$). Significant differences between monolayer and spheroid DNA quantity are indicated (*). $p = 0.0452, 0.0235, 0.0020, 0.0195$ for 24 hours, 48 hours and 96 hours, respectively $p = 0.0923$ at 120 hr.

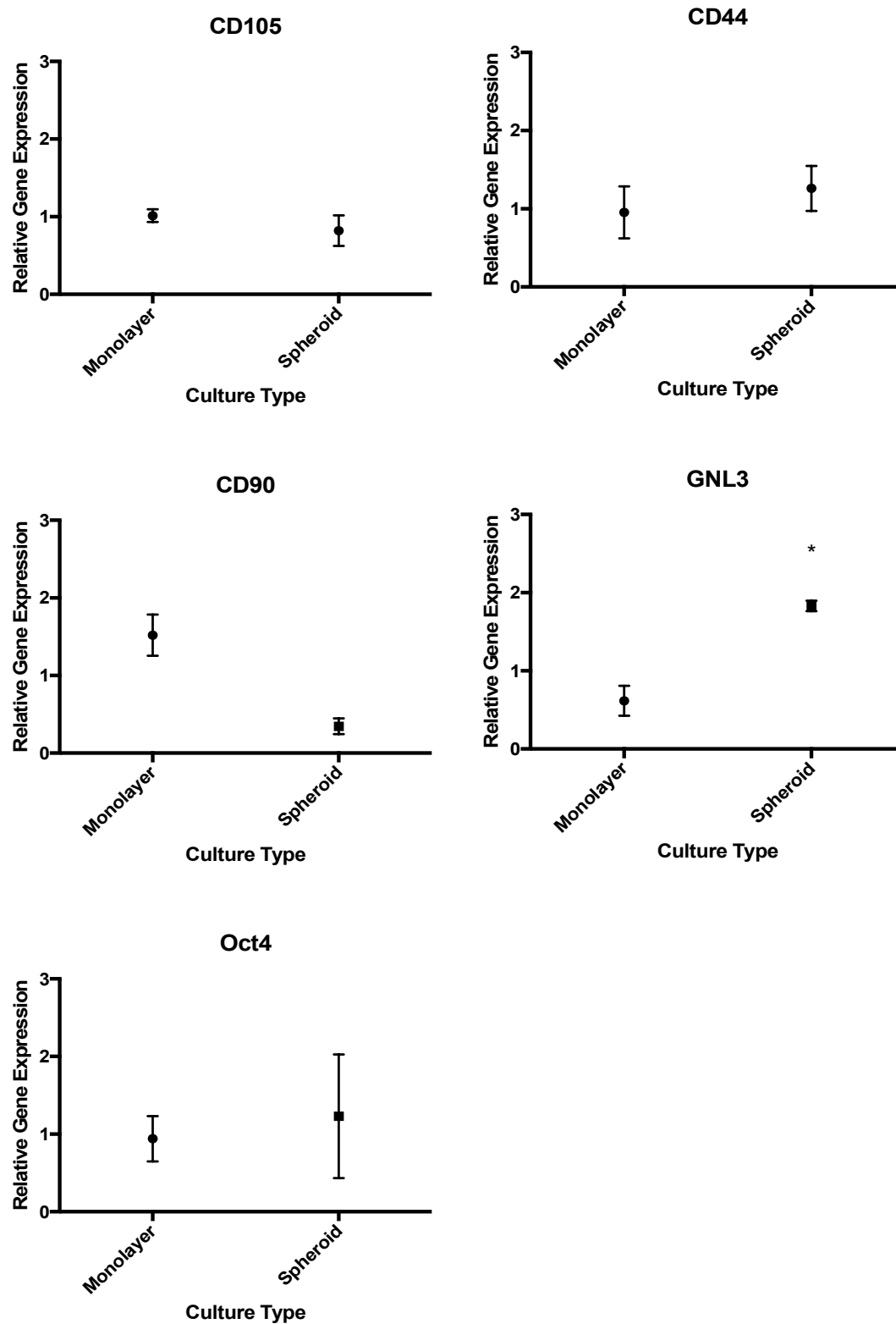


Figure 3.3 - Stem cell marker expression at 120 hours of culture monolayer vs spheroid culture. Relative gene expression was referenced to cells at 0 hours prior to culture in monolayer or spheroids. A significant difference (*) was seen in expression of GNL3 ($p = 0.0481$).

Part 2: LPS exposure:

Cell viability was unchanged with LPS stimulation for monolayer and spheroid groups, respectively. Cell viability of spheroid and monolayer cultures was not significantly different after 24 hours of LPS stimulation ($p = 0.4226$). Overall, cell viability ranged from 97-98%.

IL-6: Culture condition and LPS stimulation significantly affected IL-6 gene expression (<0.0001) and protein production was increased by culture condition ($p = 0.0016$). Culture condition was a significant effect on gene expression at 0 ($p = 0.001$), 6 ($p = 0.0025$) and 24 hours ($p = 0.0149$), whereas LPS stimulation was significant at 6 ($p = 0.0025$) and 24 ($p = 0.0205$) hours with a trend at 12 hours ($p = 0.074$).

IL-6 gene expression differed in all groups compared to monolayer control (Fig 3.5: MLPS $p = 0.0215$, $p = SC$ 0.0009, SLPS $p < 0.0001$). Spheroids treated with LPS were significantly different than all other groups (MLPS $p = 0.0006$, SC $p = 0.0140$), this was seen at 6 hours (MC $p = 0.0013$, MLPS $p = 0.0252$, SC $p = 0.0254$), but at 24 hours was only different to the monolayer control ($p = 0.0124$). Protein production by spheroid culture with or without LPS stimulation was significantly different compared to all monolayer groups overall (with LPS $p = 0.0224$; without LPS $p = 0.0189$, respectively) and at 0 hours ($p = 0.0331$).

PGE₂:

PGE₂: PGE₂ production was significantly affected by spheroid culture and LPS stimulation ($p = 0.0024$, 0.0348, respectively). Spheroid culture significantly increased PGE₂ production at 0 hours ($p = 0.0401$), but was not significant at other time points. Pairwise comparisons showed that overall spheroids cultured with or without LPS had significantly different PGE₂ production compared to monolayer controls ($p = 0.0014$ and $p = 0.0265$, respectively), but not monolayers with LPS.

mPGES-1: Gene expression of mPGES-1 by culture condition was dependent on LPS stimulation ($p = 0.0433$); and both significantly altered mPGES-1 gene expression ($p < 0.001$ and $p = 0.0006$, respectively). Pairwise comparisons showed that spheroids with LPS stimulation expressed significantly different levels of mPGES-1 than other groups (Figure X $p = MC < 0.001$, MLPS 0.0003 and SC 0.0017, respectively). Spheroid with LPS was higher than monolayer

control at 6 hours ($p = 0.0289$) and 24 hours ($p = 0.0320$), but spheroid culture was not higher than monolayers with LPS.

SDF-1: Time and culture condition significantly affected SDF-1 gene expression ($p = 0.0163$ and $p < 0.0001$, respectively) and showed dependency on each other ($p = 0.007$). Spheroid culture also significantly affected SDF-1 protein production ($p = 0.0094$) overall and at 0 hours ($p < 0.0001$), 6 hours ($p = 0.0001$), 12 hours ($p = 0.0125$) and 24 hours ($p < 0.0001$). Spheroid culture with or without LPS stimulation had significantly altered SDF-1 gene expression levels compared to monolayer cultures ($p < 0.0001$). This was evident at 0 hours ($p < 0.0001$) and 6 hours (MC vs SC 0.0016, MC vs SLPS 0.0050, MLPS vs SC 0.0074, MLPS vs SLPS 0.0278), however not at 12 hours or 24 hours. LPS stimulation was not a significant effect on SDF-1 gene expression or production.

TSG-6: Both spheroid culture and LPS stimulation altered expression of TSG-6 overall ($p = 0.0001$ and 0.0283 , respectively). Spheroid culture significantly altered TSG-6 gene expression at 0 hours ($p < 0.0001$), but was not significant at other time points. Pairwise comparisons showed spheroid culture had significantly different levels of TSG-6 than monolayer cultures at 0 hours ($p < 0.0001$) and overall (MC vs SC 0.0101, MC vs SLPS 0.0003).

IL-10: Although it appeared that spheroids from one cell line altered protein production and had higher gene expression of IL-10, due to variation between cell lines there were no significant differences detected between monolayer and spheroid for IL-10 gene expression or protein production (Fig. 3.4).

IFN γ : No measureable levels were produced or expressed by any of the cell lines.

TNF α : No measureable levels were produced or expressed by any of the cell lines.

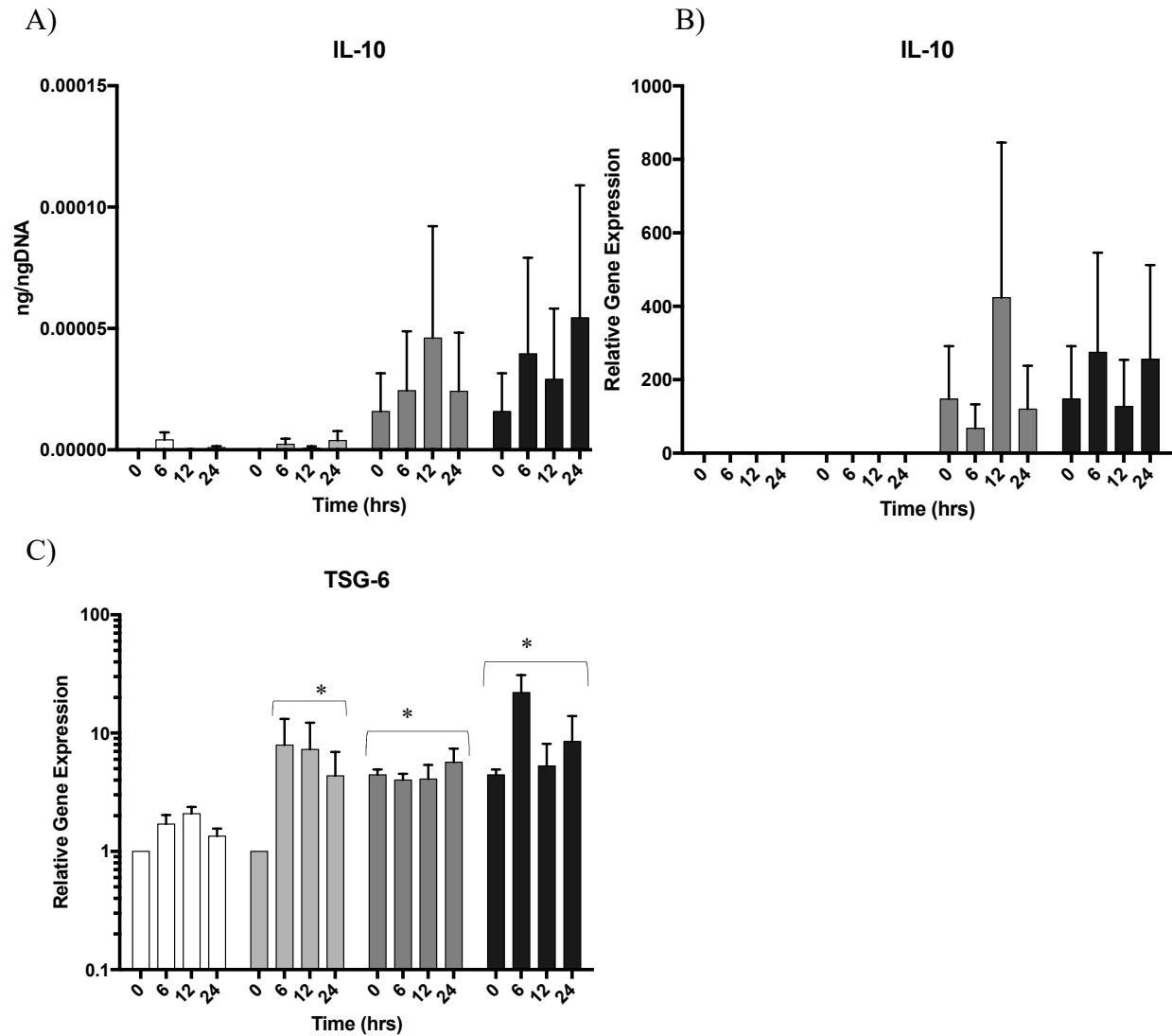


Figure 3.4 – IL-10 and TSG-6 sph vs. mono

Anti-inflammatory mediator secretion (A) and gene expression (B,C) compared to monolayer control. Monolayer control, Monolayer LPS, Spheroid, Spheroid LPS. Significant differences denoted by asterisks (*, p value ≤ 0.05)

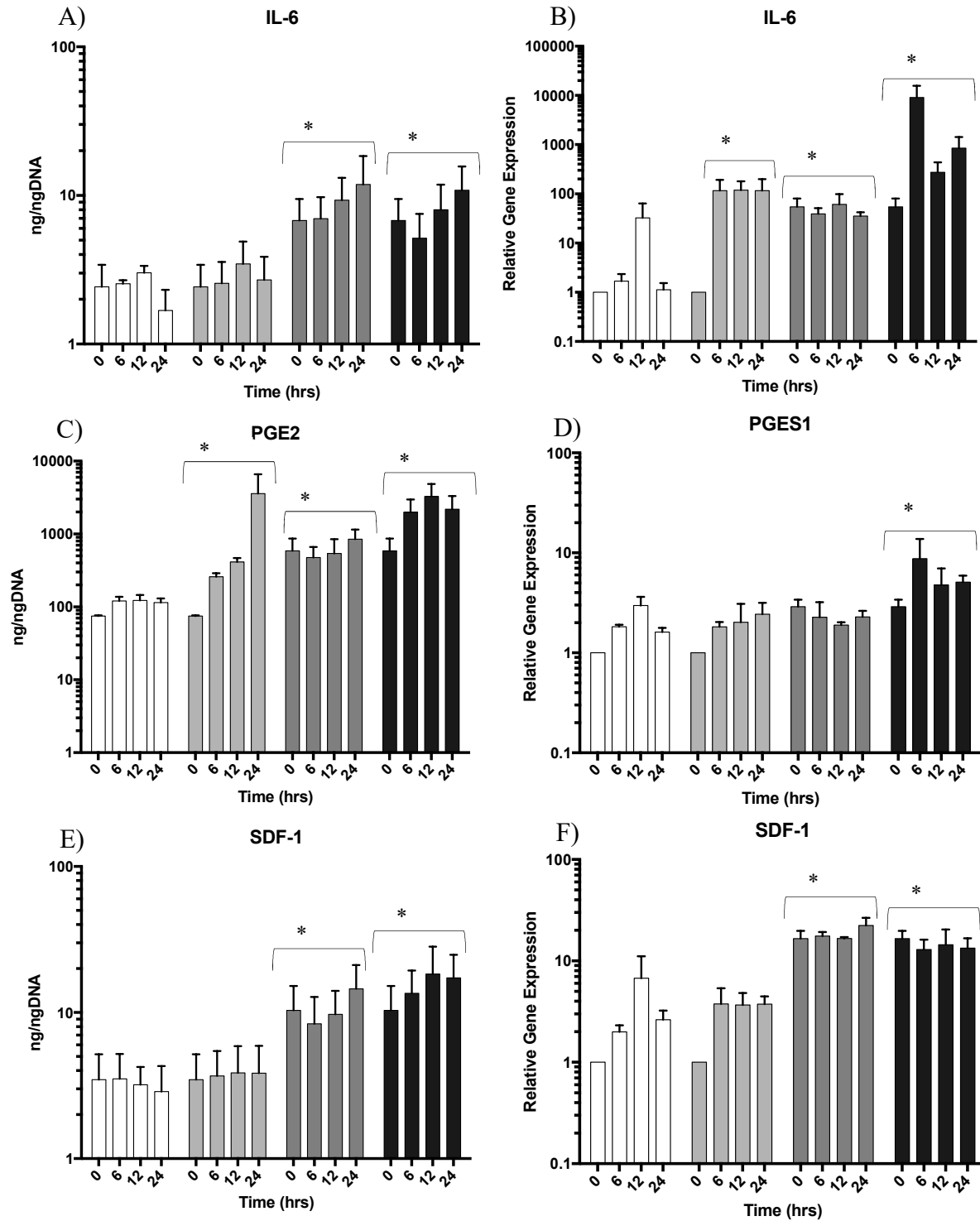


Figure 3.5 – IL-6, PGE₂, mPGES-1 and SDF-1 sph vs. mono

Anti-inflammatory mediator secretion (A, B, C) and gene expression (D, E, F) compared to monolayer control. Monolayer control, Monolayer LPS, Spheroid, Spheroid LPS. Significant differences denoted by asterisks (*, p value ≤ 0.05)

Discussion

This study identifies key characteristics of eBMSC spheroids relevant to their potential therapeutic application. First, although eBMSCs can spontaneously form spheroids they do not increase in DNA quantity like monolayer cells, making them more suitable as an end stage product rather than for cell propagation. Second, spheroid culture moves eBMSCs toward an immunomodulatory and anti-inflammatory phenotype by positively influencing IL-6, PGE₂, SDF-1 and TSG-6, but does not increase pro-inflammatory cytokines TNF α and IFN γ . This “cytokine licensing” effect occurs without the need for exogenous inflammatory stimulation. Lastly, this study features individual variation in IL-10 response to spheroid culture, which could be studied further to detect cell lines that have superior anti-inflammatory attributes.

Cell proliferation in spheroid culture has been shown to be minimal in previous studies [635,646]. Although it was demonstrated that the proportion of human BMSCs in G2/S phase of the cell cycle was similar in monolayer vs. spheroid culture, the length of time in G1, or proportion entering G0 was not investigated [635]. Additionally, [646] reported reduced cell yield at harvest compared to initial seeding cell number. As in previous studies using human BMSCs, we subjectively saw that eBMSC spheroidal cells were subjectively smaller than monolayer cells [2,3]. We also noticed that the majority of cells form a tight three-dimensional sphere with a halo of a few dissociated cells. Due to the small cell size and that not all cells enter the compact body of the spheroid, we hypothesize that loss of the dissociated cells causes a minor reduction in total DNA yield over 120 hours. Our spheroid harvest protocols for centrifugation were validated to collect all spheroidal cells during harvest. A limitation is that DNA quantity alone, rather in combination with a metabolic assay for proliferation, such as the MTT assay, was used as a measure of alterations in cell biomass. However, direct comparison of MTT assay between monolayer and spheroid cells could be misleading given differences in genes that determine metabolic rate between the culture techniques [635]. Given the poor potential for propagation, we recommend that eBMSCs are expanded in monolayer culture prior to spheroid production. As we demonstrated that spheroids altered cytokine profile, the use of spheroid culture as a manipulation to enhance therapeutic properties is their main utility.

This study is the first to validate the spheroidal culture of eBMSCs. Alteration in over 710 genes occurs with spheroidal culture of human BMSCs. The same study showed that up-regulated genes were grouped into genes associated with anti-oxidant activity, anti-neoplasia and cell cycle regulation [635]. We demonstrated that stem cell markers remained unchanged with spheroid culture except for GNL3. GNL3, also called nucleostemin, controls the G1/S transition, where either under or over expression cause G1 cell cycle arrest [677]. This finding could contribute to the discrepancy in DNA quantity between monolayer and spheroid cells in this study. A limitation is that stem cell marker gene expression was used alone, rather than in combination with flow cytometry; however, dissociation of spheroids for flow cytometry requires enzymatic dissociation which could potentially alter cell surface markers, additionally qPCR allowed assessment of Oct4, which is not on the cell surface [637]. qPCR has been found as a suitable alternative to flow cytometry when cross reactive anti-bodies are not available [499].

This study is the first to investigate alterations in cytokine profile of spheroid-cultured eBMSCs and demonstrates “cytokine licensing” by spheroid culture through enhanced production and expression of key immunomodulatory cytokines. Specifically, spheroid culture enhances IL-6 and SDF-1 regardless of inflammatory stimulation. Additionally, SDF-1 is produced in a time-dependent manner. In contrast, both spheroids and monolayer cells produce PGE₂ and TSG-6 under inflammatory stimulation; however, spheroids have enhanced PGE₂ and TSG-6 in the absence of exogenous inflammatory stimulation. A visual summary is given in Table 3.1.

Table 3.1 - Visual summary of increase in immunomodulatory mediators compared to monolayer cells not treated with LPS (monolayer control/MC)

	Spheroid (S)	LPS (MLPS)	Spheroid LPS (SLPS)
IL-6	↑		↑↑
PGE ₂	↑	↑	↑↑
SDF-1	↑		↑
TSG-6	↑	↑	↑
IL-10	Individual variation – spheroid increased in one cell line		
IFN γ	No production		
TNF α	No production		

“Cytokine licensing” is the process by which stem cells become activated into an anti-inflammatory state in the presence of certain inflammatory conditions including addition of

activated PBMCs, LPS, or inflammatory cytokines $\text{TNF}\alpha$, IL-1 β or IL-17 [496,678]. Under these conditions stem cells produce enhanced levels of PGE_2 , TSG-6 and sometimes IL-1ra or IL-10 which induces an anti-inflammatory IL-10 phenotype in macrophages and modulates T cells, dendritic cells and neutrophils [4,214,496,679]. Attempts to increase therapeutic potential of monolayer cells have been made through the addition of $\text{TNF}\alpha$, IL-1 β or nitric oxide [672]. However, Ylöstalo, et al. [4] demonstrated that spheroid culture can activate human BMSCs in a similar manner to when MSCs are in an inflammatory environment, and this results in enhanced peripheral blood IL-10 production and survival in murine peritonitis [3]. Similar to this study, [4] found that spheroids were “self-activated” to produce PGE_2 and TSG-6 and this profile was able to decrease proliferation of macrophages, and enhance their production of IL-10 and IL-1ra. A limitation of this study is that a co-culture model to assess the anti-inflammatory effects of spheroids was not performed. However, the profile of increased PGE_2 , TSG-6 and IL-6 has since been used to validate the use of different culture media to produce spheroids with an anti-inflammatory phenotype [2,4,5,646].

We have successfully demonstrated a novel technique to enhance the therapeutic anti-inflammatory potential of eBMSCs. Spheroid manipulation of eBMSCs has potential to be a superior therapeutic modality over monolayer culture due to immediate anti-inflammatory activity of spheroids, a stable anti-inflammatory profile despite the presence or absence of environmental inflammatory mediators and reduces likelihood of an adverse reaction to residual exogenous stimulating cytokines. Such changes are hypothesized to be due to the altered cell-cell and cell-ECM interactions, cell morphology, and oxygen and nutrient gradients in aggregates [680,681]. Recently, two studies found functional secretion of PGE_2 , TSG-6 and IL-6 by human BMSC aggregates is dependent on caspase dependent IL-1 signaling [2,5]. Peak of IL-1 signaling and gene expression of cytokines from spheroids is at 72 hours of spheroid culture, and if activation of IL-1 signaling is inhibited, spheroids are unable to cause macrophages to change to an anti-inflammatory phenotype that produces IL-10 and low $\text{TNF}\alpha$ [2]. Such an alteration in the phenotype of eBMSCs warrants investigation in equine models of disease prior to clinical use, additionally, the production of spheroids needs to be optimized to allow high-throughput production needed for equine clinical application.

We were unable to reach conclusions about the spheroid production of IL-10 due to individual variation. It has been established that PGE₂ is the main cytokine produced by MSCs to have an immunomodulatory effect on peripheral blood mononuclear cells, however, in the absence of PGE₂ IL-10 can be produced [4,209,560,561,669]. Only subsets of individuals, and in fact a subset of cultured cells, produce IL-10 as their main inflammatory mediator [561]. A similar cell-line-dependent production of IL-1ra was demonstrated in the treatment of mice with acute lung injury, where select clonal populations of murine MSCs [562] directly produced IL-1ra. The production of IL-10 by certain donors in place of, or as well as, PGE₂ could explain the variation seen in both the ELISA results of IL-10 and PGE₂ in this study. IL-10 production by eBMSC spheroids warrants further investigation due to the direct anti-inflammatory effects of the anti-inflammatory cytokine IL-10.

Conclusion

We have demonstrated that equine bone marrow derived stem cells spontaneously form spheroids in hanging drop culture and maintain excellent viability over 120 hours and maintain most stem cell markers compared to monolayer culture. Compared to monolayer culture, cell mass in spheroid culture does not increase so likely utilization is for direct therapeutic application rather, than culture propagation. Spheroid culture produces differential expression of a range of immunomodulatory factors compared to monolayer culture, which is often independent of LPS exposure or time. Such modulation without the need to introduce exogenous biomaterials or cytokines may make spheroid culture a feasible approach for manipulation of eBMSCs prior to therapeutic use. Studies that investigate the influence of spheroids on equine disease states, as well as optimization for clinical applications are warranted.

CHAPTER 4

Feasibility of Needle Injection and High-throughput Culture of Equine Bone Marrow Derived Stem Cells as Three-Dimensional Spheroids

Abstract

Equine bone marrow derived mesenchymal stem cells (eBMSCs) are currently used to treat equine musculoskeletal disease; however, results in clinical models of musculoskeletal inflammation, like synovitis and osteoarthritis, have had variable efficacy. We demonstrated that three-dimensional spheroid culture of eBMSCs induced anti-inflammatory properties compared to traditional monolayer culture. For clinical relevance, spheroids would need to be viable after injection through needles, and a high-throughput technique for culture would be needed. This study aimed to investigate the effects of needle passage on viability and senescence in spheroid and monolayer cultures, and compare two methods of high-throughput culture with a previously used hanging drop technique. We demonstrated that spheroids maintained significantly higher viability and lower senescence than monolayer cultures after needle passage. Spheroids successfully formed in both methods of high-throughput culture. The high-throughput methods had significantly smaller spheroid size than hanging drop culture; however, this did not affect the expression of the anti-inflammatory phenotype markers, prostaglandin E synthase 2, TSG-6 and IL-6. In fact, one high-throughput culture method significantly increased TSG-6 production and may have increased therapeutic potential due to the direct anti-inflammatory effects of TSG-6.

Introduction

eBMSCs are used for musculoskeletal soft tissue injury in horses [464,518]. However, variations in processing and injection technique contribute to variation in stem cell efficacy [682], thus far disappointing results have been found for eBMSC treatment of equine inflammatory musculoskeletal disease like synovitis [455] and osteoarthritis [447]. Chapter 3 indicates that three-dimensional culture of eBMSCs in spheroids alters their immunomodulatory profile, leading to a similar anti-inflammatory phenotype to human BMSC spheroids that produce high levels of PGE₂, TSG-6 and IL-6 relative to adherent monolayer cultured BMSCs [2-4,646].

eBMSCs maintain >99% viability for up to 120 hours in spheroidal culture and maintain stem cell markers CD104, CD90, CD44 and Oct4. The benefit of spheroid culture over monolayer culture is that spheroids have increased production of PGE₂, TSG-6 and IL-6 regardless of stimulation with lipopolysaccharide. In other models, phenotype is effective at changing macrophages from an inflammatory phenotype that produces high TNF α , to an anti-inflammatory phenotype that is low in TNF α and high in IL-10 [4,646], and can modulate murine peritonitis [3]. Our previous data used uniform 50,000 cell spheroids made in Bioperfecta™ 96-hanging drop plates; however, previously used treatment doses for horses range from 5-20 million cells per injection site [683]. Therefore, this culture technique and other methods of hanging drop culture need to be replaced by high-throughput culture for clinical applications.

The effect of needle bore size on viability has been investigated for equine, murine and human BMSCs but not spheroids or spheroidal eBMSCs [684-686]. Equine BMSC injection is performed with 22g [610,687], 20g [688] or 18g needles with internal diameters of 413 μ m, 603 μ m and 838 μ m, respectively. The diameter of Bioperfecta 50,000 cell spheroids in our previous experiment was approximately 300-400 μ m so it is unknown if needle passage will be detrimental to cell viability. The first objective was to determine the effect of needle use and bore size on the viability of eBMSCs and to compare spheroid and monolayer cell viability after injection through needles. The second objective was to develop a culture technique that would allow high-throughput culture of eBMSC spheroids without significantly altering the gene expression profile for markers of an activated phenotype (TSG-6, PGE₂ and IL-6) or stem cell markers (CD105 and Oct4). For the first objective, it was hypothesized that cells injected through needles would have increased senescence and lower viability than control cells, and that spheroid culture would lower viability and increase senescence of injected cells. For the second objective, it was hypothesized that there would be no difference in the gross spheroid size between two types of commercially available non-adherent culture flasks, that the size of spheroids formed in non-adherent flasks would be different to hanging drop spheroids and that high-throughput spheroids would have no difference in gene up-regulation compared to monolayer culture of the anti-inflammatory and immunomodulatory factors TSG-6, IL-6 and

prostaglandin E synthase 2 (PTGES2); or the stem cell markers CD-105 and Oct4 compared to hanging drop spheroids.

Materials and methods

Needle injection

Cell culture: A previously characterized eBMSC cell line determined to undergo tri-lineage differentiation and to be Oct-4 and CD90 low and MHCII negative by flow cytometry was used. Cells were cryopreserved in liquid nitrogen vapor, then were recovered and expanded as monolayer cultures in low-glucose GlutaMax DMEM with 10% fetal bovine serum and incubated at 37° C, 5% CO₂, and 90% humidity, with trypsinization of passage 2 eBMSCs at 70% confluence. eBMSCs were washed thoroughly in PBS and cultured as monolayers in adherent tissue culture flasks, or as 50,000 cell spheroid cultures using a hanging drop technique [648] for 72 hours. Culture media comprised of low-glucose GlutaMax DMEM with 110µg/mL sodium pyruvate (Gibco) plus 10% horse serum (Gibco) and 100U/mL sodium penicillin, 100µg/mL streptomycin sulfate (Sigma) was used and eBMSCs were incubated at 37° C, 5% CO₂, and 90% humidity. At 72 hours, spheroids were harvested and monolayer cells were trypsinized and washed in PBS.

Needle injection: Photomicrographs were taken of spheroid cells during spheroid formation, prior to needle passage and of a subset after needle passage. Horse serum (0.5mL) was used to suspend 1x10⁶/mL monolayer cells or ten 50,000 cell spheroids. Cells were drawn up in syringes and injected through 22g, 20g or 18g needles or a no needle control in triplicate at 0.25mL/second.

Viability assessment: After injection, spheroids were dissociated with Liberase TL (Roche) to form a single cell suspension. The dissociated spheroid and monolayer cells were stained with calcein AM and ethidium homodimer-1 for quantitative viability assay using a hemocytometer. Cell senescence was determined using a β-galactosidase enzyme assay (ThermoFisher) on monolayer cells, dissociated spheroid cells and intact spheroids. Colony forming unit assays were performed on monolayer cells by plating serial dilutions of cells on plastic 100mm-diameter cell culture dishes (ThermoScientific) in triplicate.

Statistics: Shapiro-Wilk normality testing was performed on all data imported from Microsoft Excel. To assess the effect of needle gauge on cell viability and senescence within culture type, one-way analysis of variance (ANOVA) was performed and Dunnett's *post hoc* test used to compare each gauge size to the needleless control. Differences between monolayer and spheroid culture under each needle gauge condition were tested with Student's t test. For monolayer colony forming unit data, a one-way ANOVA was performed to assess the effect of needle bore size with Dunnett's *post hoc* test to compare to the control. All analysis was performed with SAS JMP® Pro 11.0.0. Significance was set at $p \leq 0.05$, mean $\pm$ S.D. is reported, unless stated otherwise.

High-throughput culture

Cell culture: Three eBMSC cell lines collected from horses with IACUC approval were previously determined to undergo tri-lineage differentiation and to be Oct-4 and CD90 low and MHCII negative by flow cytometry then cryopreserved, were used. Cells were recovered from cryopreservation and expanded in monolayer, with media supplementation every 48 hours. Pilot studies showed that 2×10^6 cells/mL seeding density for high-throughput spheroid formation was optimum, passage 4 cells were seeded at this density in either Corning or Nunclon ultra low-adherent T75 cell culture flasks, 10mL of eBMSC containing media was added initially, with addition of 10mL at 48 hours of culture. Passage 4 hanging drop spheroids of 50,000 cells were also made in Biopreacta™ 96-hanging drop plates for comparison and monolayer cells in 24 well plates (50,000 cells/ well). All MSCs were grown in culture media comprised of low-glucose GlutaMax DMEM with 110µg/mL sodium pyruvate (Gibco) plus 10% MSC FBS (Gibco) and 100U/mL sodium penicillin, 100µg/mL streptomycin sulfate (Sigma) and incubated at 37° C, 5% CO₂, and 90% humidity for 72 hours.

Microscopic Image Analysis: Phase contrast microscopic images of 50 spheroids per treatment group were taken. ImageJ was used to assess spheroid area and longest diameter at 72 hours.

High-throughput spheroid harvest: The high-throughput flask spheroids were gently suspended and 6mL aliquots were removed that were either unfiltered or filtered into three size ranges (<100µm, 100-300µm and >300µm) for comparison of cell number (DNA quantity) (Quant-IT™, Invitrogen™), and for RT-qPCR of a known size for high-throughput techniques.

RT-qPCR: After media removal from monolayer cells and centrifugation of harvested spheroids, MSCs were lysed with Trizol reagent and stored at -80°C until RNA isolation. Quantitative RT-PCR (qPCR) utilizing pre-designed (TSG-6, IL-6) or custom (PTGES2, CD105, Oct-4) TaqMan primers and MGB probes were used to assess expression of phenotype activation and stem cell markers (Tables 4.1, 4.2). RNA was isolated using Trizol, and purified using a commercially available column-based protocol (RNeasy, Qiagen) and included an on-column DNase treatment to exclude genomic template contamination. First strand complimentary DNA (cDNA) was synthesized by use of oligo(dT) priming and a commercial reverse transcriptase kit; non reverse transcriptase controls were produced (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA). Complimentary DNA was amplified by fluorescent real time PCR (Absolute Quantitative PCR: ABI PRISM 7300 Sequence Detection System, Applied Biosystems, Foster City, CA) using custom or pre-designed primers and MGB probes. For custom sets, sequences were obtained from published literature, designed to span exon-exon boundaries using available sequence data on GenBank (Primer Express Software Version 3.0, Applied Biosystems, Foster City, CA) and were cross-referenced against NIH Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/>). Relative gene expression was calculated using the comparative threshold cycle method ($\Delta\Delta C_t$) using monolayer cells as the reference. GAPDH RNA expression was used as an endogenous control, after verifying gene expression stability in monolayer and spheroid eBMSC samples compared to other potential endogenous controls (B2M, RPL13A, RPL32, HPRT1) using GeNorm algorithm normalization.

Table 4.1 - Applied Biosystems Pre-designed Primer-Probe Sets

Gene	Product ID
IL-6	Ec03468679_m1
TSG-6	Ec03468339_m1

Table 4.2 - Custom Primer-probe sequences:

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')
Oct-4	CAGCTCGGGCTCGAGAAG	TTCTGGCGACGGTTGCA	ACGTGGTACGAGTGTGG-6FAM
CD105	TCCACATCCTCTTCCTGGAGTT	GGACCTTTGGATAGTCAGCTTCA	CCAAGGGATGTGTCAGAG-6FAM
PTGES2	TGCCCCAGGAAGGAGACA	GGGCGCTGATGATGACAGA	TGCAACAACCTGAATGAC-6FAM
GAPDH	CAAGTTCCATGGCACAGTCAAG	GGCCTTCCGTTGATGACAA	CCGAGCACGGGAAG-6FAM

Statistics: Power analysis using G*Power (Dusseldorf), based on our previous data of gene expression between monolayer and spheroid cultured cells, found that a sample size of 3 cell lines was needed to detect a 4-fold difference in gene expression with 80% power. Data was recorded in Microsoft Excel and analyses were performed with SAS JMP® Pro 11.0.0. Shapiro-Wilk normality testing was performed on all data, to choose the appropriate parametric/ non-parametric comparisons. Logarithmic gene expression data was assessed by repeated measures one-way ANOVA. Dunnett's multiple comparisons were compared to Bioperfecta control if significance was found. Spheroid size data was assessed by Kruskal-Wallis and Kolmogorov-Smirnov tests. A priori significance was set at $p \leq 0.05$. Graphical data is presented as mean $\pm$ S.E.

Results

Needle injection

Monolayer percentage viability was $78\% \pm 10\%$ for control, $80\% \pm 3\%$ for 18g, $83\% \pm 1\%$ for 20g and $80\% \pm 5\%$ for 22g, with no difference between needle sizes and the control. Spheroid cultured MSCs were $367.9\mu\text{m} \pm 13\mu\text{m}$ at 72 hours of culture and no resistance was experienced when injecting through any needle sizes. Percentage viability for spheroid cells was $93\% \pm 6\%$ for control, $93\% \pm 4\%$ for 18g, $96\% \pm 1\%$ for 20g and $96\% \pm 1\%$ for 22g, with no significant difference between needle sizes and the control. However, there was a significant difference between spheroid and monolayer viability for 22g ($p = 0.020$) 20g ($p = 0.00020$) and 18g ($p = 0.012$) needles, with spheroids having higher viability. There was no difference in viability between the monolayer and spheroid controls ($p = 0.11$).

Monolayer 22g cells had significantly lower senescence than control cells ($p = 0.049$). There were no significant differences within the dissociated spheroid group. Whole spheroid 20g cells had significantly lower senescence than controls ($p = 0.028$). Control monolayer cells had significantly higher senescence than dissociated ($p = 0.011$) or whole control spheroids ($p = 0.013$), with no difference between whole or dissociated spheroids ($p = 0.98$). Similarly, 20g monolayer cells had significantly higher senescence than whole ($p = 0.0035$) or dissociated ($p = 0.0041$) 20g spheroids. There was significantly higher senescence of 18g monolayer cells than

whole spheroids ($p = 0.0062$), but there was no difference between monolayer and dissociated spheroid cells ($p = 0.13$) (Fig. 4.1).

Monolayer colony forming units were 44 ± 13 for control, 29 ± 4 for 18g, 36 ± 7 for 20g and 35 ± 9 for 22g. There were no significant differences compared to the control (Fig. 4.2).

High-throughput culture

eBMSCs aggregated into spheroids over 72 hours using Biopreacta, Corning and Nunclon ultra low adherence culture vessels. Subjectively, the time to aggregation was similar, by 24 hours spherical aggregates were seen in each group (Fig. 4.3). Spheroid area and size measurements are summarized in Table 4.3. Compared to Biopreacta spheroids, Corning and Nunclon flask spheroid area and diameter were significantly different in cell lines from all horses and combined (Fig. 4.4). Within each cell line there was no difference in diameter and area between Corning and Nunclon spheroids, but when data was combined a significant difference was seen for diameter ($p = 0.007$) and area ($p = 0.01$). Even so, the proportions of spheroids in each size range were not significantly different for combined data ($<100\mu\text{m}$ $p = 0.497$, $100-300\mu\text{m}$ $p = 0.235$, $>300\mu\text{m}$ $p = 0.535$, Fig 4.5) between Corning and Nunclon culture vessel groups.

There were no significant differences between high-throughput flask culture methods and Biopreacta spheroids for gene expression of IL-6, PTGES2, CD105 and Oct4. However, culture in Nunc flasks produced significantly higher TSG-6 ($p = 0.036$, Fig. 4.6).

Table 4.3 - Area and diameter measurements $\pm$ S.E. for spheroids produced by Biopreacta hanging drop or two types of low adherent flasks combined results from 3 horse cell lines.

Within each parameter, significant differences between culture types are indicated by the same letter ($p \leq 0.05$)

Culture method	Diameter (μm)	Area (μm^2)
Biopreacta Hanging drop (control)	345 ± 5.9 ^{a,b}	$84,796 \pm 2,301$ ^{a,b}
Corning T75 Flask	95 ± 3.6 ^{a,c}	$7,840 \pm 637$ ^{a,c}
Nunclon T75 Flask	118 ± 4.9 ^{b,c}	$11,214 \pm 1,052$ ^{b,c}

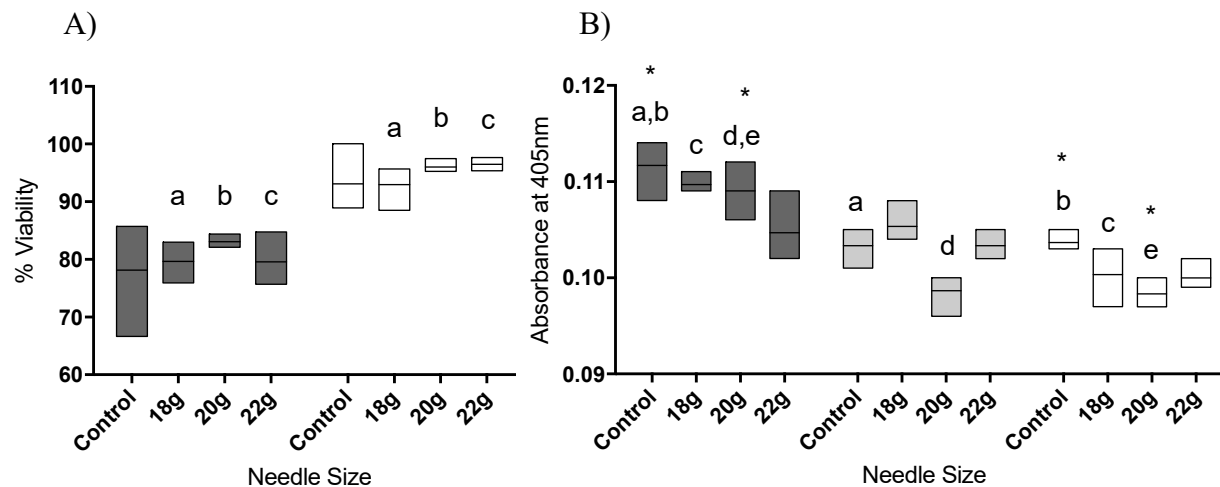


Figure 4.1 – Viability and senescence after needle passage

A) Percentage viability determined by live-dead counts. B) Absorbance of β -galactosidase, increased absorbance indicates higher senescence. The same letters indicate significant differences between monolayer and spheroid samples, asterisks indicate differences within culture type ($p \leq 0.05$). Monolayer (■); spheroid (□); dissociated spheroid (◐)

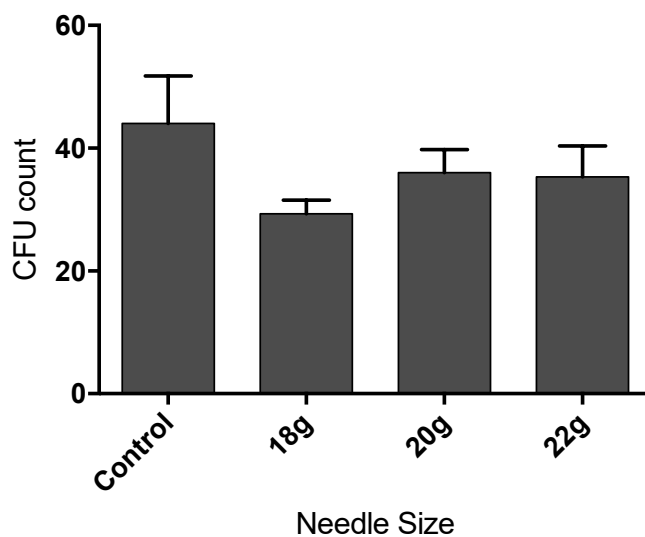


Fig. 4.2 – Monolayer colony forming units

Colony forming unit (CFU) count after 10 days of culture for monolayer control and after passage through 18g, 20g or 22g needles. There were no significant differences in CFU count between any of the needle sizes and control.

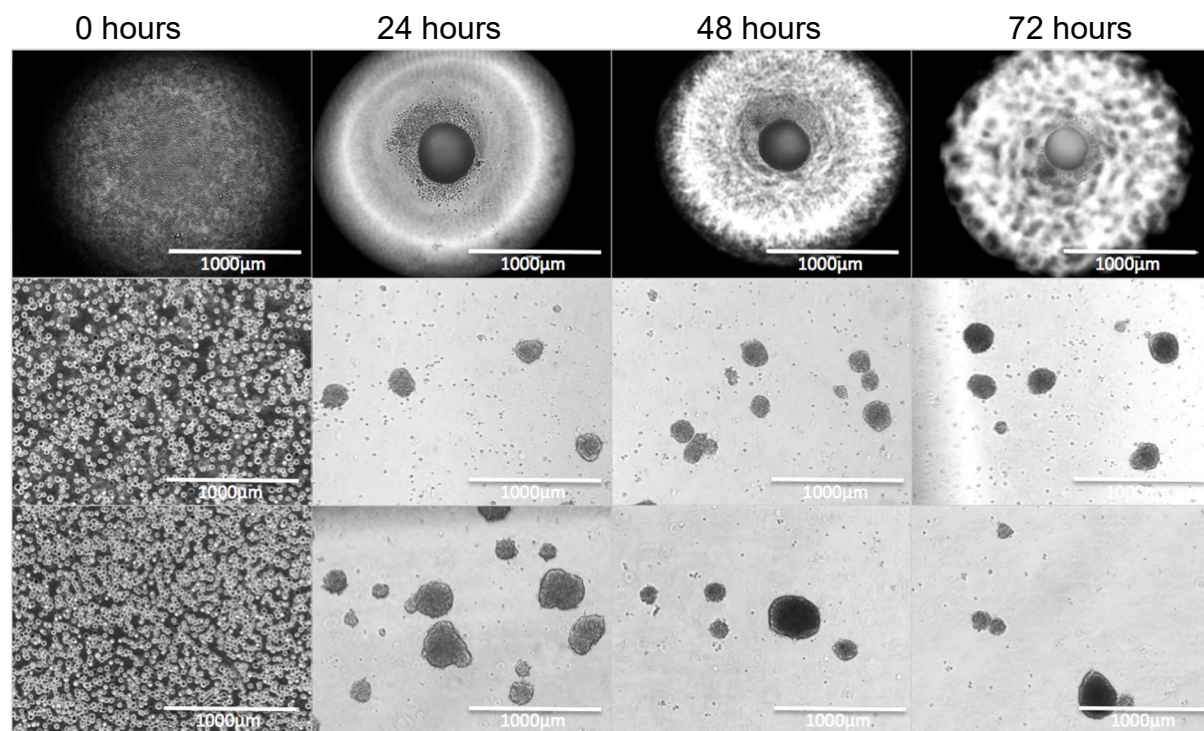


Figure 4.3 – High-throughput spheroid aggregation

Horse 1 cells forming spheroids from 0 to 72 hours in hanging drop (top), Corning flask (middle), Nunclon flask (bottom). Scale bar is 1000µm.

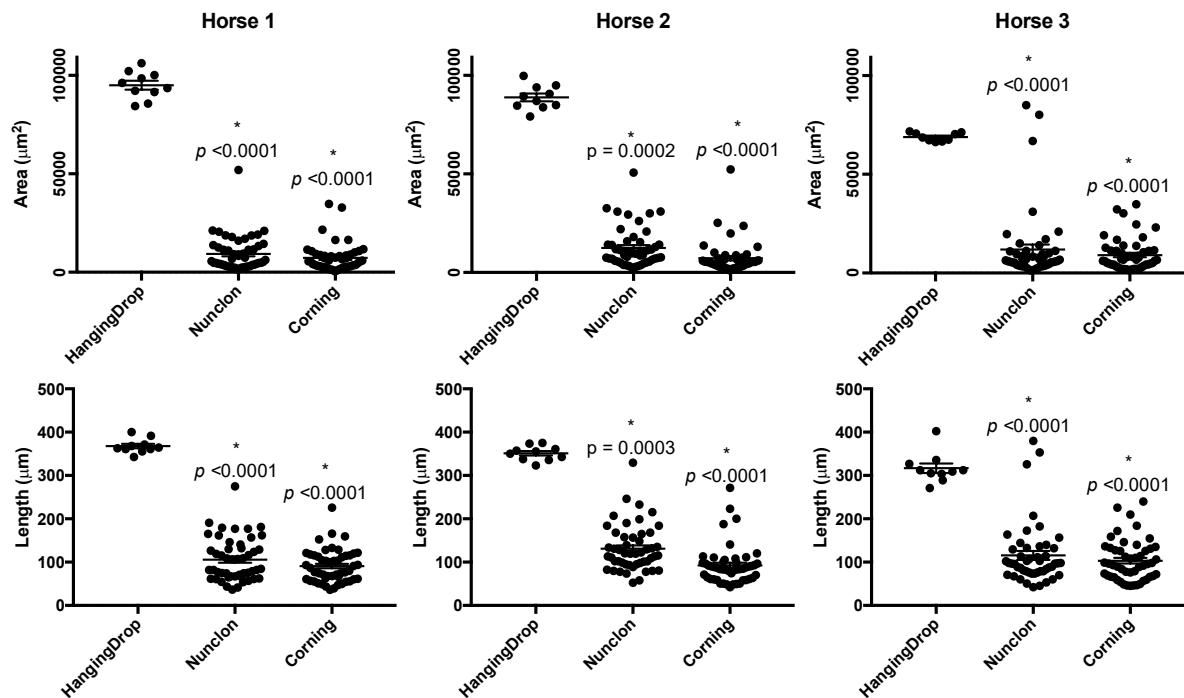


Figure 4.4 – Spheroid size as area and diameter length

There was a significant difference in spheroid area (top) and diameter (bottom) between hanging drop and Corning or Nunclon spheroids. Nunclon and Corning were not significantly different. Asterisks show significant difference compared to hanging drop control ($* p \leq 0.05$).

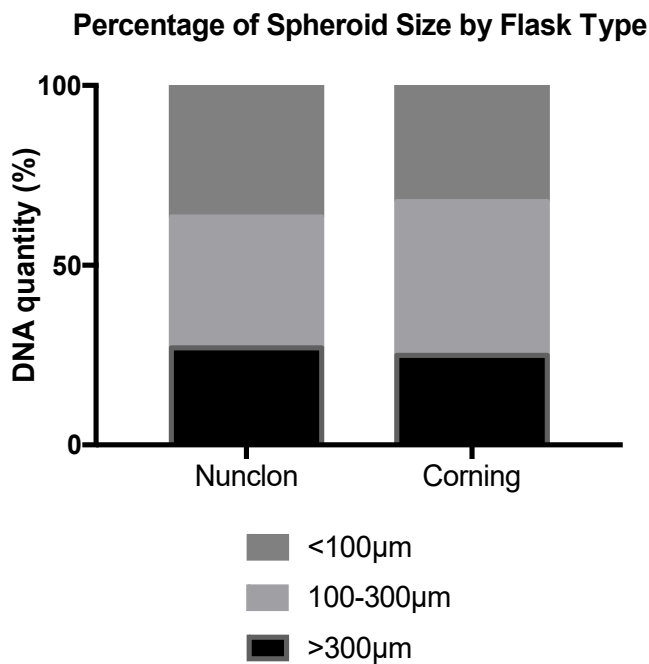


Figure 4.5 – Size ranges of high-throughput culture

There was no significant difference in the proportions of spheroids in each size range between Nunclon and Corning T75 flasks

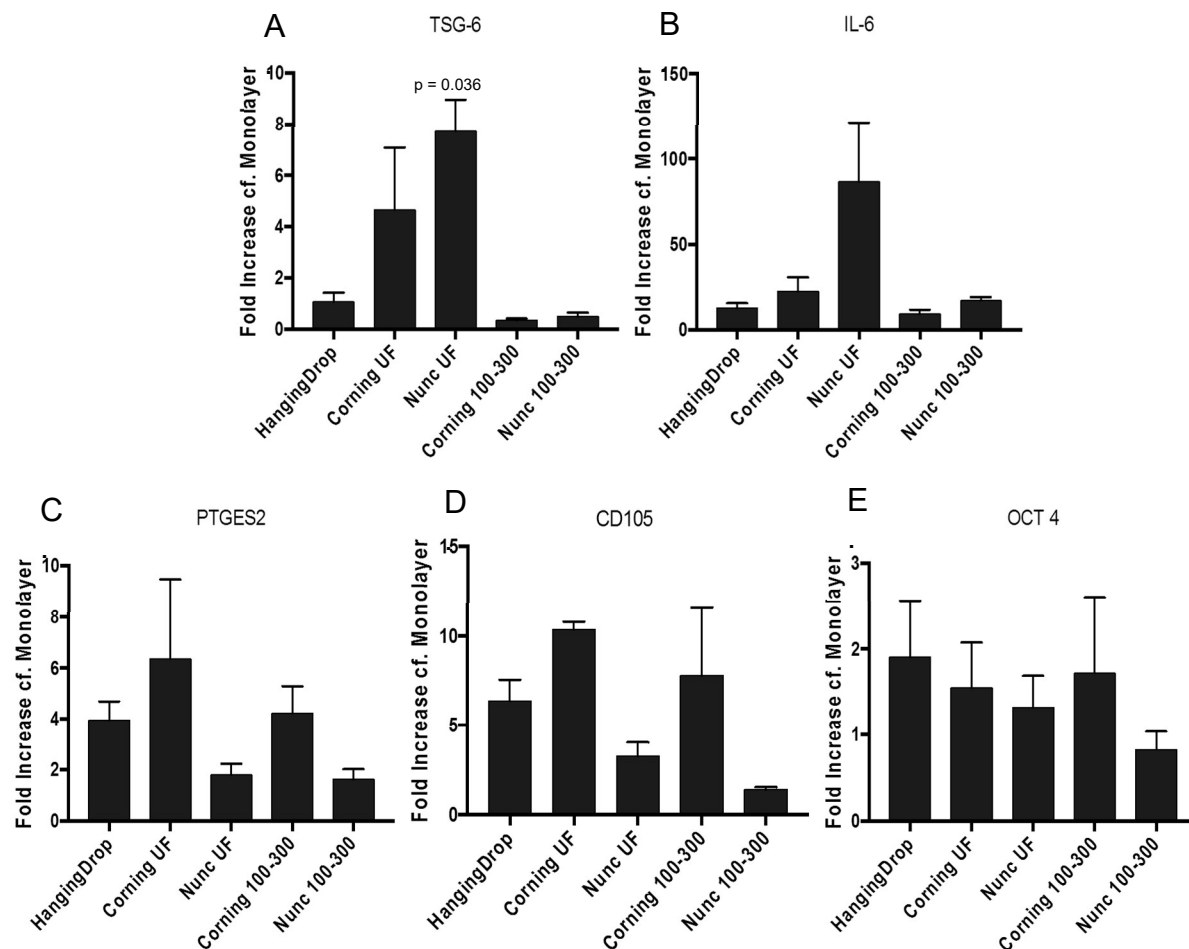


Figure 4.6 – Gene expression of anti-inflammatory phenotype markers by high-throughput culture

There were no significant differences in fold increase of gene expression compared to monolayer controls between hanging drop and Nunclon or Corning flasks (unfiltered or 100-300 μ m) for IL-6, PTGES2, CD105 and OCT4. There was a statistical difference for the anti-inflammatory protein TSG-6 in Nunclon unfiltered spheroids compared to hanging drop ($p=0.036$).

Discussion

The study in chapter 3 demonstrated that eBMSCs are able to form three-dimensional spheroids that have high viability and express markers associated with an anti-inflammatory phenotype similar to what has been reported for human BMSC spheroids [2-4]. In this study, we investigated the feasibility of using eBMSC spheroids clinically by assessing viability post-injection and evaluating a high-throughput culture method. We demonstrated that passage through bore size of needles commonly used in clinical eBMSC applications does not significantly affect either monolayer or spheroid cell viability. Surprisingly, spheroids have significantly increased viability and lower senescence than monolayer cells under most injection conditions. We also demonstrated that high-throughput production of eBMSC spheroids is possible with two different types of commercially available ultra-low adherent flasks, with similar production of spheroids 100-300 μ m. Additionally, we found that culture using Nunclon flasks results in elevated levels of the anti-inflammatory protein, TSG-6, which counteracts IL-1 β and TNF α [6]. This information enables practitioners to use their choice of 18g, 20g or 22g needles without detrimental effects on eBMSC viability, and gives researchers valuable information on the characteristics of high-throughput cultured spheroids for clinical application. Furthermore, higher viability and lower senescence rates of spheroids under the conditions used in this study could be therapeutically beneficial due to increased metabolically active cells and fewer senescent cells which, importantly, are known to secrete matrix metalloproteinases and inflammatory cytokines [689,690].

This study showed that spheroids had significantly higher viability than monolayer cells when passed through 18g, 20g or 22g needles. Although there was a trend toward a difference between control spheroid and monolayer cell viability, this did not reach significance. Garvican, et al. [684] demonstrated reduction in monolayer eBMSC viability immediately after injection through 21g and 23g 50mm long needles and 2 hours after injection through 19g needles. It was speculated that increased shear stress from smaller bore needles caused immediate cellular damage. Given that spheroidal cells contain a large cell mass that is protected within the center of the spheroid, relatively less exposure to shear forces may have resulted in the differences in viability between monolayer and spheroid. A limitation of this study was that cell viability was

not assessed at multiple time points following injection; however, CFU analysis of monolayer cells showed no differences after injection through different needle sizes.

All cells were suspended in 100% horse serum as an injection vehicle, which was allogeneic compared to the cells. Serum is used as an injection vehicle in our clinic and has been reported by others [683], so it was used to simulate a clinical situation. eBMSCs stored at 4-8°C in allogeneic serum have a similar viability to other suspension mediums at 12 and 24 hours, but viability decreased over time [684]. eBMSCs in this study were exposed to allogeneic serum for relatively shorter periods of time; however, it is possible that cell viability was affected. Relatively less exposure of the cell mass to the suspension medium may have resulted in increased viability of spheroid culture. Comparison of spheroid eBMSCs to monolayer eBMSCs in a range of suspension would be needed to determine the effects of injection vehicle on monolayer and/or spheroid eBMSCs.

Whole spheroids had lower levels of cellular senescence than monolayers for the control, 18g and 20g samples, and in the control and 20g samples for dissociated spheroids. Human BMSCs in monolayer do not express telomerase and undergo senescence in 30-40 population doublings as the telomeres shorten [493,494]. Similarly, eBMSCs show consistent senescence by 27 population doublings, which is approximately the 10th passage [517]. In this study, eBMSCs used to make monolayers and spheroids for needle injection were at passage 3 and remained in culture for 72 hours; therefore, the number of population doublings was low. However, there are significant differences between the ability of monolayer and spheroid BMSCs to proliferate. Several studies have found that when BMSCs have aggregated to form spheroids, they lose proliferative capacity [2,4,635]. We suspect they may arrest at the G1-S transition based on up-regulation of nucleostemin (GNL3) found in Chapter 3. Similarly, we found that DNA quantity in spheroids did not increase, but it did in monolayer culture, suggesting that population doublings ceased in the spheroid-cultured group. Cessation of population doublings in spheroid culture may delay senescence and result in lower β -galactosidase absorbance. However, decreased senescence could be a property inherent to spheroid culture and unrelated to population doublings. For example, human adipose derived MSC (AdMSC) spheroids cultured on chitosan films had less senescence at higher population doubling rates than monolayer

AdMSCs [691]. Thorough investigation of senescence in eBMSC spheroid culture requires population-doubling controls to elucidate the mechanism behind the findings of this study.

High-throughput flask culture of human periosteal MSC spheroids has been investigated for mass production [692]. Non-adherent flasks have a hydrophobic surface, which makes cells more attracted to hydrophilic surfaces or each other [693]. Different hydrophobic surfaces for spheroid culture of murine embryonic stem cells found that surface chemical properties affect the ability of cells to aggregate, the proportion of spheroids 100-300 μ m, and spheroid size uniformity [650]. Spheroid diameters of <300 μ m are optimal because oxygen and nutrient diffuse to a maximum of 150-200 μ m, with a necrotic core resulting in cell masses >500 μ m [694]. Additionally, large spheroid diameter may limit the ability of spheroids to be passed through a broader range of needle sizes, as 22g needles are 413 μ m internal diameter. In contrast, the diameter of both high-throughput spheroid culture techniques is significantly lower and more variable than the Bioperfecta hanging drop technique. This is likely because the Bioperfecta spheroids are made from 50,000 cells/ spheroid but the high-throughput techniques allow cells to self-aggregate with fewer, and more variable, cells per spheroid. Despite these differences, most assessed gene expression levels were not different between Bioperfecta and high-throughput cultures, indicating that variability of spheroid size may not affect spheroid functional properties. However, TSG-6 did differ, which could be due to alterations in spheroid size in the Nunclon Flask, or other biochemical interactions between the cell and the surface coating.

This study is the first to compare gene expression differences between high-throughput culture techniques and the hanging drop technique. Methods of making spheroids include hanging drop culture, low attachment or micropatterned culture flasks, or dynamic culture [648,649,652,654,692]. The studies that have assessed the anti-inflammatory properties of BMSC spheroids to date have used hanging drop culture [2-4]. A recent study using a spinning bioreactor to culture human AdMSC spheroids demonstrated these spheroids significantly increased survival from acute hepatitis in a murine model to 75% compared to 58% survival with monolayer treatment [695]. BMSC spheroid studies have found that PGE₂, TSG-6 and IL-6 are increased when MSC phenotype becomes anti-inflammatory [2-5]. We have demonstrated that gene expression of prostaglandin E synthase, TSG-6 and IL-6 are still up-regulated in spheroids produced by high-throughput culture. In fact, the expression of TSG-6 in Nunclon cultured

spheroids is around 8-fold higher than hanging drop spheroids. As TSG-6 is directly anti-inflammatory, high-throughput culture in this manner could be therapeutically advantageous.

A key consideration for the eventual use of BMSC spheroid culture for clinical trials is the time and relative ease of production. The traditional hanging drop culture technique using drops of media suspended on an “agar lid” is time consuming and can result in wastage of spheroids. Although development of specialized hanging drop techniques, such as the Bioperfecta hanging drop plates decreases wastage, they take longer to seed and harvest spheroids compared to using a non-adherent flask. Additionally, seeding and harvest of hanging drop cultures requires all trays to be opened fully and harvest requires additional steps and equipment when pooling to get large quantities. Increased air-culture interface and additional steps may increase exposure of the culture to contamination. Another practical consideration is that stem cell culture laboratory technicians are familiar with T75 flask handling and aseptic technique for clinical use, compared to other techniques of spheroid culture. A limitation of this study in regard to practical use, is that needle passage was not performed with high-throughput cultures. However, as Nunclon and Corning flask spheroids are significantly smaller than Bioperfecta spheroids, we predict that they would easily pass through all needle sizes investigated.

Conclusion

MSC viability is not affected by needle size when 18g, 20g or 22g needles are used. However, single cells in suspension have less cell viability and increased cell senescence compared to spheroids. This could be due to fewer cells exposed to cytotoxic culture or injection vehicle substrates when in spheroid form, or be an inherent characteristic of spheroid culture. More research is needed to ascertain if viability and senescence is altered when other injection vehicles or culture media are used, and if increased viability and decreased senescence of spheroid culture contributes to therapeutic benefit.

Nunclon and Corning flask methods of high-throughput spheroid production produce smaller spheroids than Bioperfecta hanging drop production, and an equivalent proportion of spheroids that are <100µm, 100-300µm and >300µm. Despite the size disparity between the hanging drop and high-throughput techniques, high-throughput spheroid production via Nunclon or Corning T75 flasks is comparable to hanging drop spheroid culture to maintain up-regulation of anti-

inflammatory and immunomodulatory genes compared to traditional monolayer culture. For continuing research, Nunclon flasks could be used because the enhanced expression of the anti-inflammatory protein TSG-6 may give therapeutic advantages when spheroids are used to treat inflammation.

CHAPTER 5

Spheroids Cultured From Different Allogeneic Cell Lines Behave Differently in Inflammatory Conditions

Abstract

Spheroid cultured human BMSCs have shown enhanced anti-inflammatory potential and we recently demonstrated similar findings for equine BMSCs (eBMSCs). Although eBMSCs have been used successfully in some equine orthopedic diseases, their efficacy in OA remains disappointing. To enhance the therapeutic potential of eBMSCs in OA, determination of the influence of spheroid culture, IL-1 β stimulation and cell line on their anti-inflammatory phenotype is needed. This study aimed to investigate differences in gene expression of key markers of an anti-inflammatory phenotype between spheroids from different cell lines with or without inflammatory stimulation with IL-1 β . Another aim was to determine differences between monolayer and spheroid cells from different cell lines under IL-1 β stimulation. eBMSCs in monolayer or spheroid culture from 6 cell lines were stimulated with 10ng/mL IL-1 β for 24 hours, then relative gene expression of TSG-6, mPGES-1, PTGES2, IL-10 and IL-6 were determined by qPCR. Cell line was a significant determinant of gene expression and determined the extent that spheroids responded to IL-1 β stimulation and alterations between monolayer and spheroid culture. Key cell lines with potentially enhanced therapeutic potential as spheroids were identified that could be investigated for ability to ameliorate *in vivo* inflammation.

Introduction

eBMSCs have been used successfully to treat musculoskeletal diseases like superficial flexor tendonitis [464] and intra-articular soft tissue injury [277]. The former study found that eBMSC treatment of core tendon lesions reduced the re-injury rate from ~55% to 25%, highlighting their regenerative and trophic properties [464]. However, there has been less success for treatment of equine osteoarthritis (OA) whose development and continuation relies on a cycle of inflammation and tissue breakdown.

OA is a degenerative disease of joints, affecting approximately 85% of aged horses [25]. Inflammation is central to the initiation and propagation of OA, best exemplified by the pathophysiology of post-traumatic OA (PT-OA), which affects horses; approximately 2% of racehorses sustain joint injury each month leading to wastage [24]. Joint trauma causes inflammation of all joint tissues leading to a peak of inflammatory cytokines including IL-1 β and TNF α within 24-48 hours of injury [173,175,696]. IL-1 β stimulates chondrocytes and synoviocytes to produce catabolic enzymes like matrix-metalloproteinases (MMPs) that breakdown articular cartilage, increases inflammatory cytokines including IL-1 β , TNF α , IL-6 and IL-8, and induces chondrocyte apoptosis [697]. A peak of chemokines follows IL- β and TNF α in a murine model of PT-OA, which contributes to increased macrophages, CD4 $^{+}$ and CD8 $^{+}$ T cells and activated B cells in the synovium [173,189]. Macrophages are drivers for OA inflammation and are increased in 76% of patients with symptomatic OA [216,698]. Cartilage breakdown products act as danger associated molecular patterns (DAMPs) that stimulate receptors of immune cells to activate NF κ B, enhancing cytokine transcription [178,699]. Chondrocyte apoptosis and limited ability for cartilage repair lead to permanent damage following inflammation in PT-OA [147,700,701]. Therefore, mitigation of inflammation is a key goal to prevent and reduce detrimental tissue effects.

Mesenchymal stem cells (MSCs) are immunomodulatory and anti-inflammatory, trophic, anti-fibrotic and chemoattractant [702]. They have disease-modifying effects for PT-OA in non-equine species caused by soft tissue (rabbit, goat) and osteochondral (mouse) injuries [438,572,703]. MSCs have increased efficacy when injected early after ACL transection in rabbits or after collagenase injection in mice [572,704]. This is in part because MSC anti-inflammatory activity is enhanced by high IL-1 β concentrations in vitro, and likewise by high levels of synovial inflammation and DAMPs in serum in murine models of OA [209,705]. Given the relatively high levels of initial inflammation in PT-OA, and evidence that early MSC injection is effective, we suspect that induction to an anti-inflammatory phenotype by inflammatory cytokines with subsequent modulation of inflammation is an integral mechanism of MSCs in ameliorating PT-OA.

Three-dimensional spheroid culture of BMSCs activates them into an anti-inflammatory phenotype that is able to increase IL-10 and decrease TNF α from inflamed macrophages [4].

Hallmarks of this phenotype are increases in BMSC expression and secretion of IL-6, tumor necrosis factor α inducible protein 6 (TSG-6) and PGE₂ [2-4]. The cause of this phenotypic shift is activation of IL-1 and Notch signaling [2], which is caspase dependent and linked to the contraction of actin, a component of the cytoskeleton, as spheroidal cells aggregate and contract [5]. IL-6 and PGE₂ are immunomodulatory cytokines with complex actions, capable of both promotion and amelioration of inflammation depending on what pathway is activated, or how much is produced, respectively [192]. TSG-6 is a small protein with direct anti-inflammatory action by ameliorating the effects of IL-1 β and TNF α [6]. Paracrine effects of PGE₂ and TSG-6 produced by MSCs decrease proliferation of immune cells and reduce inflammation in *in vitro* and *in vivo* models of sepsis, peritonitis, corneal injury and arthritis [209,560,669,670,706]. Additionally, the anti-inflammatory cytokine IL-10 has been produced by some MSCs [561], and was produced by one cell line in our previous study in response to spheroid culture, but not in other cell lines. Although it is clear that production of immunomodulatory cytokines can vary greatly with spheroid culture, IL-1 β stimulation or the specific cell line investigated, the influence on these factors on BMSC activity has not been performed for any species. Knowledge of how eBMSCs react to these variables may help improve anti-inflammatory efficacy in OA.

The primary aim of this study was to investigate individual variation in the expression of key markers indicating an anti-inflammatory phenotype between spheroids from eBMSC cell lines isolated from different horses, with or without IL-1 β stimulation. The second aim was to determine differences between monolayer and spheroid cells from these cell lines stimulated with IL-1 β . It was hypothesized that there would be differences in expression of TSG-6, prostaglandin E synthase 1 (mPGES-1), prostaglandin E synthase 2 (PTGES2), IL-6 and IL-10 between spheroids made from different horse cell lines, and that spheroid culture would have greater expression of these genetic markers than their monolayer equivalent with variation between horses.

Materials and methods

Cell culture: Six eBMSC cell lines were isolated from 6 horses with IACUC approval and cryopreserved in liquid nitrogen vapor. Cells were recovered from cryopreservation, expanded as

monolayer cultures and trypsinized at 70% confluence. Passage 3 cells were cultured as monolayer, 500,000 cells in 24 well adherent tissue culture flasks and allowed to adhere for 36 hours, or as 50,000 cell spheroid cultures using a hanging drop technique and allowed to aggregate for 72 hours [648]. Quadruplicate wells of monolayer and quadruplicate groups of 10 spheroids for each cell line and treatment were created. All MSCs were grown in culture media comprised of low-glucose GlutaMax DMEM with 110µg/mL sodium pyruvate (Gibco) plus 10% MSC FBS (Gibco) and 100U/mL sodium penicillin, 100µg/mL streptomycin sulfate (Sigma) and incubated at 37° C, 5% CO₂, and 90% humidity for 72 hours.

IL-1β stimulation: Spheroids were harvested into polypropylene tubes in groups of 10 after 72 hours of aggregation, as described by Kelm, et al. [648]. Monolayer cells were passaged routinely. All cells were centrifuged and washed in PBS then transferred into each well of a 24 well non-adherent (spheroid) or adherent (monolayer) culture plate. Culture media consisting of low-glucose GlutaMax DMEM with 110µg/mL sodium pyruvate (Gibco) plus 10% MSC FBS (Gibco) and 100U/mL sodium penicillin, 100µg/mL streptomycin sulfate (Sigma) with or without 10ng/mL recombinant IL-1β (R&D Systems) [315,421,707] was added to each well (500µL). Each treatment condition was created in quadruplicate for each cell line of the monolayer and spheroid samples, respectively. eBMSCs were stimulated for 24 hours then media was aspirated from monolayer wells, spheroids were harvested by centrifugation.

Expression of stem cell surface markers: Stimulated and unstimulated monolayer and spheroid cells in quadruplicate were lysed with Trizol reagent and stored at -80°C until RNA isolation. Quantitative RT-PCR (qPCR) utilizing pre-designed (TSG-6, IL-6, IL-10) or custom (mPGES-1, PTGES2) TaqMan primers and MGB probes was used to assess expression of phenotype activation (Table 5.1, 5.2). RNA was isolated using Trizol, and purified using a commercially available column-based protocol (RNeasy, Qiagen) and included an on-column DNase treatment to exclude genomic template contamination. First strand complementary DNA (cDNA) was synthesized by use of oligo(dT) priming and a commercial reverse transcriptase kit; non reverse transcriptase controls were produced (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA). Complementary DNA was amplified by fluorescent real time PCR (Absolute Quantitative PCR: ABI PRISM 7300 Sequence Detection System, Applied Biosystems, Foster City, CA) using custom or pre-designed primers and MGB probes. For custom sets, sequences

were obtained from published literature, designed to span exon-exon boundaries using available sequence data on GenBank (Primer Express Software Version 3.0, Applied Biosystems, Foster City, CA) and were cross-referenced against NIH Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/>). Relative gene expression was calculated using the comparative threshold cycle method ($\Delta\Delta C_t$) using GAPDH RNA expression as an endogenous control, after verifying gene expression stability in monolayer and spheroid eBMSC samples compared to other potential endogenous controls (B2M, RPL13A, RPL32, HPRT1) using GeNorm algorithm normalization.

Table 5.1 - Applied Biosystems Pre-designed Primer-Probe Sets

Gene	Product ID
IL-10	Ec03468646 ml
IL-6	Ec03468679 ml
TSG-6	Ec03468339 ml

Table 5.2 - Custom Primer-probe sequences:

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')
mPGES-1	AGGGTGTGTTGCGATTTCTTTT	GGGCACCGAGCATAATGC	CGGCCGGAGTTCA-6FAM
PTGES2	TGGCCCAGGAAGGAGACA	GGGCGCTGATGATGACAGA	TGCAACAACCTGAATGAC-6FAM
GAPDH	CAAGTTCCATGG CACAGTCAAG	GGCCTTTCGGTT GATGACAA	CCGAGCACGGGAAG-6FAM

Statistics: Raw C_t data for each target was normalized to the endogenous control (GAPDH), previously found to be stable under spheroid culture conditions via GeNorm. Gene expression of spheroids treated with or without IL-1 β and monolayer cells treated with IL-1 β were compared to the mean of untreated monolayer cells of the given cell line to calculate fold increase gene expression using Livak's ($\Delta\Delta C_t$) method. Fold increase was converted to a logarithmic scale and tested for normality with Shapiro-Wilk normality test. A 2 way ANOVA was performed in JMP® Pro 13.0.0 (SAS Institute Inc.) to elucidate the effects of IL-1 β treatment and horse/ cell line on gene expression of IL-6, TSG-6, mPGES-1, PTGES2 and IL-10 by spheroid cultures; or between culture type (spheroid vs. monolayer) and horse for IL-1 β treated spheroid and monolayer cultures. If no significant interactions were encountered, post hoc Tukey HSD pairwise comparisons were performed on significant main effects, simple effects were investigated further if significant interactions were encountered. A priori significance was set at $p \leq 0.05$.

Results

Either spheroid culture or IL-1 β exposure induced changes in gene expression compared to monolayer control. Additionally, significant individual variation was seen between cell lines under each type of condition for all genes.

IL-6: IL-6 gene expression increased compared to monolayer control in response to spheroid culture, IL-1 β exposure, or both spheroid culture plus IL-1 β exposure. The greatest up-regulation was in IL-1 β stimulated spheroids followed by IL-1 β stimulated monolayers (Fig. 5.1).

Responses of spheroids to IL-1 β stimulation: Horse cell line ($p < 0.0001$) and IL-1 β treatment ($p < 0.0001$) were significant effects for IL-6 up-regulation by spheroids, suggesting that both are important determinants of spheroid IL-6 gene expression. Spheroids from horse 1 were significantly different than from horse 3 ($p = 0.035$) and 5 ($p = 0.049$); horse 5 from horse 2 ($p = 0.024$), 3 ($p < 0.0001$) 4 ($p = 0.017$), and 6 ($p = 0.041$); and horse 3 from horse 6 ($p = 0.043$). These results suggest that, overall, spheroids from horse 5 eBMSCs up-regulate IL-6 the least and horse 3 eBMSCs up-regulate IL-6 the greatest, compared to most other cell lines; however, IL-6 expression is increased in all cell lines by exposure to IL-1 β .

Spheroid vs. monolayer with IL-1 β stimulation: When IL-1 β stimulated monolayer and spheroid cells were compared; there were significant effects of both cell line ($p < 0.0001$) and culture method ($p < 0.001$), with a significant interaction between them ($p < 0.0001$). This suggests that the degree of IL-6 up-regulation in response to IL-1 β stimulation is dependent on what cell line is responding, and if the cells are in spheroid or monolayer form. Simple effects of cell line within each culture type showed that spheroids stimulated with IL-1 β from horse 5 were significantly different than horse 1 ($p = 0.035$), 2 ($p = 0.0018$) and 3 ($p = 0.0002$), and horse 4 from horse 3 ($p = 0.011$). Thus, horse 5 had the lowest and horse 1, 2 and 3 had the higher IL-6 levels as IL-1 β stimulated spheroids, which agrees with the overall expression by spheroids. In contrast monolayer cells stimulated by IL-1 β from horse 1 differed from all other cell lines ($p < 0.0001$) except for horse 5, which differed from horses 2, 4 and 6 ($p < 0.0001$). Horse 2 and 6

also differed ($p = 0.0015$). Thus, horse 1 and 5 had the highest expression and horse 6 had the lowest expression.

TSG-6: There was a high degree of TSG-6 gene up-regulation by some cell lines, but a down-regulation by others in spheroid culture without IL-1 β ; however, with IL-1 β stimulation there was up-regulation for both spheroid and monolayer culture types (Fig. 5.1).

Responses of spheroids to IL-1 β stimulation: There were significant effects of horse cell line ($p < 0.0001$) and IL-1 β treatment ($p < 0.0001$) on TSG-6 expression for spheroids, with a significant interaction between them ($p = 0.044$). Thus, spheroids from some cell lines responded to a different extent to IL- β stimulation than others. For non-stimulated control spheroids, horse 6 was significantly different than horses 1, 2 ($p < 0.0001$) and 5 ($p = 0.0097$); horses 3 and 4 from horses 1 and 2 ($p < 0.0001$); and 5 from horse 1 ($p < 0.0001$) and 2 ($p = 0.0002$). Thus, control spheroids for horse 3, 4 and 6 have the highest expression of TSG-6 and horse 1 and 2 have the lowest levels. For IL-1 β stimulated spheroids horse 6 continued to be significantly different from horses 1, 2 ($p < 0.0001$), and 5 ($p = 0.0006$); and horses 3 and 4 from horses 1 and 2 (3 vs. 1 $p = 0.0005$, 3 vs. 2 $p = 0.022$, 4 vs. 1 $p = 0.0004$, 4 vs. 2 $p = 0.018$); however, horses 1 and 2 had up-regulated expression to a greater extent than horse 5 resulting in no significant difference between them. With IL-1 β treatment, horses 3, 4 and 6 still have the highest expression of TSG-6, but horse 1 and 2 increased enough to be equivalent to horse 5.

Spheroid vs. monolayer with IL-1 β stimulation: Horse cell line ($p < 0.0001$) and culture type ($p = 0.006$) significantly affected TSG-6 expression, with a significant interaction between them ($p < 0.0001$). As with IL-6, TSG-6 expression between spheroid and monolayer cells stimulated with IL-1 β exhibit cell-line dependent differences in the degree of response. Compared to the results for IL-1 β stimulated spheroids above where horse 6 was one of the highest TSG-6 expressing cell lines, differences in monolayer response make horses 4 and 5 differentially up-regulated compared to other cell lines (3 v. 5 $p = 0.0011$, all other comparisons $p < 0.0001$). Additionally, horses 1 and 3 are different than horses 2 and 6 (1 vs. 2 $p = 0.0002$, 1 vs. 6 $p = 0.0023$, all other comparisons $p < 0.0001$). Therefore, for monolayer culture stimulated with IL-1 β the greatest up-regulation of TSG-6 is by horses 4 and 5 and the least by horses 2 and 6. Comparison of these results with IL-1 β stimulated spheroids suggests that the manipulation of

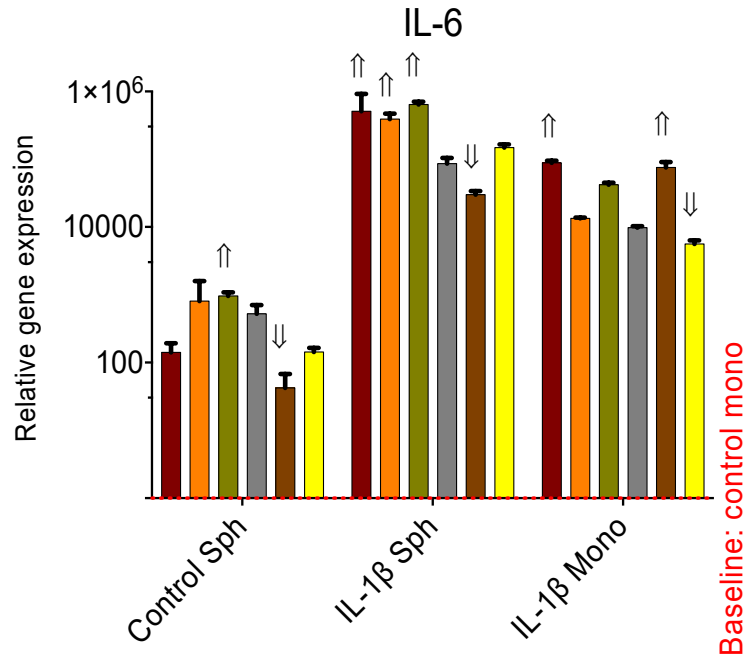
spheroid culture can accelerate the TSG-6 expression from one of the least expressing in monolayer to one of highest expressing, as is the case with cells from horse 6.

mPGES-1: mPGES-1 was up-regulated under all culture conditions stimulated with IL-1 β , but there was a combination of up- and down-regulation in spheroids not exposed to IL-1 β (Fig. 5.2).

Responses of spheroids to IL-1 β stimulation: Both cell line and IL-1 β treatment were significant effects on mPGES-1 expression of spheroids ($p < 0.0001$), with a significant interaction between them ($p = 0.001$). For control spheroids, horses 1, 2 and 3 were down-regulated and significantly different than horses 4, 5 and 6 (1, 3 vs. 4 $p < 0.0001$, 1 vs. 5 $p = 0.0004$, 1 vs. 6 $p = 0.0001$, 2 vs. 4 $p = 0.001$, 2 vs. 5 $p = 0.017$, 2 vs. 6 $p = 0.007$, 3 vs. 5 $p = 0.001$, 3 vs. 6 $p = 0.0005$); and for IL-1 β stimulated spheroids horses 1, 2, 3 and 4 became up-regulated, but remained significantly different than horses 5 and 6 (1,3 vs. 6 $p < 0.0001$, 1 vs. 5 $p = 0.0027$, 2 vs. 5 $p = 0.009$, 2 vs. 6 $p = 0.0002$, 3 vs. 5 $p = 0.0002$, 4 vs. 5 $p = 0.047$, 4 vs. 6 $p = 0.0011$). Thus, horses 5 and 6 responded to IL-1 β stimulation to a greater degree than horse 4, but remained more up-regulated than the first 3 cell lines.

Spheroid vs. monolayer with IL-1 β stimulation: There were significant effects of cell line and culture type ($p < 0.0001$), with a significant interaction between them ($p = 0.004$). Whereas in spheroid form, cell lines 5 and 6 had highest mPGES-1 expression; in monolayer culture stimulated by IL-1 β horse 5 was significantly higher than horse 6 ($p = 0.0012$), in addition to horses 1, 2, 3 and 4 ($p < 0.001$). However, horse 6 did remain up-regulated and significantly different than horses 2 ($p = 0.043$) and 3 ($p = 0.031$). Horse 6 spheroids have an enhanced ability to respond to IL-1 β by producing mPGES-1 when in spheroid form and horses 2 and 3 consistently have lower expression of mPGES-1 compared to other cell lines regardless of culture condition.

A)



B)

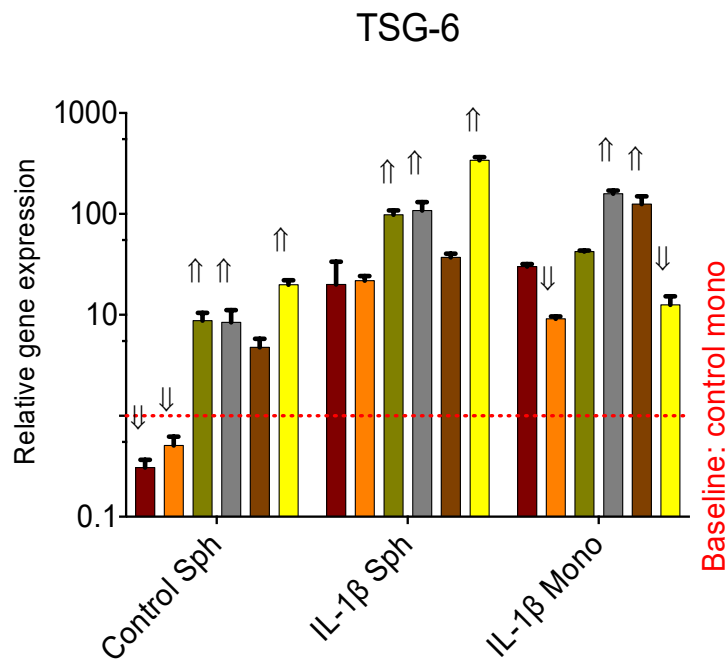


Figure 5.1 – IL-6 and TSG-6 by cell line

Relative gene expression of IL-6 (A) and TSG-6 (B) compared to monolayer cells non-stimulated with IL-1 β (monolayer control). Arrows, $\uparrow$ indicate significant differences and highly expressing cell lines, $\downarrow$ indicate significant differences and low expressing cell lines, per treatment group. Refer to the text for significant differences between cell lines with intermediate expression ($p \leq 0.05$). ■ Horse/ cell line 1; ■ Horse/ cell line 2; ■ Horse/ cell line 3; ■ Horse/ cell line 4; ■ Horse/ cell line 5; ■ Horse/ cell line 6.

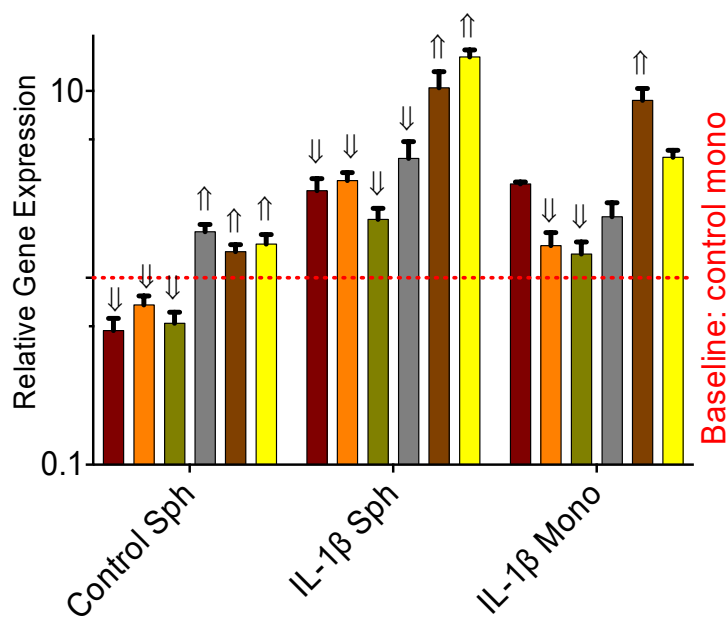
PTGES2: PTGES2 gene expression had an opposite response pattern as a result of IL-1 β stimulation compared to the other genes studied: it was mildly up-regulated in control spheroid culture but became down-regulated with exposure to IL-1 β in both spheroid and monolayer culture types (Fig. 5.2).

Responses of spheroids to IL-1 β stimulation: There were significant effects of cell line ($p < 0.0001$) and IL-1 β treatment ($p < 0.0001$) on spheroid PTGES2 expression, with a significant interaction between them ($p = 0.011$). Horse 3 was down-regulated and significantly different than horses 2 ($p = 0.032$), and 6 ($p = 0.017$); and horse 1 was significantly different with less up-regulation than horse 6 ($p = 0.040$) for control spheroids. Similarly, when spheroids were stimulated with IL-1 β , horse 1 and 3 were down-regulated and significantly different from horse 5 and 6 (1 vs. 5 $p = 0.0027$, 1 vs. 6 $p = 0.0071$, 3 vs. 5 $p = 0.0006$, 3 vs. 6 $p = 0.0017$). Although spheroids from different cell lines respond to IL-1 β by lowering PTGES2 expression to different degrees, horses 1 and 3 consistently have the lowest and horse 6 one of the highest PTGES2 expressions.

Spheroid vs. monolayer with IL-1 β stimulation: Horse cell line ($p < 0.0001$) and culture type ($p = 0.0042$) were significant effects on PTGES2 expression. Cells from horses 5 and 6 produced similar expression of PTGES2 but had significantly different increased expression compared to horses 1 (vs. 5 $p < 0.0001$, vs. 6 $p = 0.0001$), 2 (vs. 5 $p < 0.0001$, vs. 6 $p = 0.0009$), 3 (vs. 5, 6 $p < 0.0001$) and 4 (vs. 5 $p = 0.0001$, vs. 6 $p = 0.004$), which were also equivalent. Thus, culture as monolayer or spheroid will alter the way that cells respond to IL- β , however each cell line responds similarly, with cell lines 5 and 6 having relatively higher PTGES2 expression.

A)

mPGES-1



B)

PTGES2

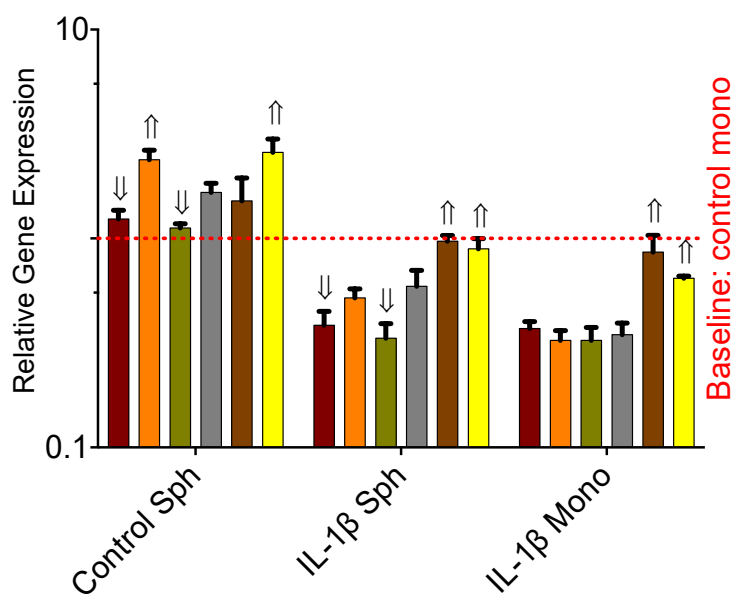


Figure 5.2 – mPGES-1 and PTGES2 by cell line

Relative gene expression of mPGES-1 (A) and PTGES2 (B) compared to monolayer cells non-stimulated with IL-1 β (monolayer control). Arrows, $\uparrow\uparrow$ indicate significant differences and highly expressing cell lines, $\downarrow\downarrow$ indicate significant differences and low expressing cell lines, per treatment group. Refer to the text for significant differences between cell lines with intermediate expression ($p \leq 0.05$). ■ Horse/ cell line 1; ■ Horse/ cell line 2; ■ Horse/ cell line 3; ■ Horse/ cell line 4; ■ Horse/ cell line 5; ■ Horse/ cell line 6.

IL-10: There was variable IL-10 expression depending on horse, culture type and IL-1 β stimulation, with some cell lines only expressing IL-10 under specific conditions (Fig. 5.3). Horse 5 had undetectable IL-10 expression under all culture and IL-1 β treatments, and was excluded from analysis. Horse 6 expressed IL-10 in both IL-1 β stimulated and non-stimulated spheroid culture, but not in either monolayer treatment group. Given the inability to calculate horse 6-fold-change using the within cell line monolayer control reference, fold-change gene expression for horse 6 data was calculated using the mean expression of all untreated monolayer cells that had produced IL-10 in order to include data for interpretation. Cell lines 1 and 2 had undetectable IL-10 expression in non-stimulated spheroid culture, but did produce IL-10 when stimulated with IL-1 β . To allow inclusion of cell lines 1 and 2 to assess the effects of cell line and IL-1 β treatment on spheroid IL-10 expression, monolayer control levels of gene expression were used in place of absent values for non-stimulated spheroids from each cell line, respectively.

Responses of spheroids to IL-1 β stimulation: The effects of cell line ($p < 0.0001$) and IL-1 β treatment ($p < 0.0001$) were significant for IL-10 expression of spheroids, with a significant interaction between them ($p < 0.0001$). As expected for control spheroids, cell lines 3, 4 and 6 were significantly different compared to horses 1 and 2 ($p < 0.0001$); cell line 4 was significantly higher than cell line 3 ($p = 0.004$). When spheroids stimulated with IL-1 β were considered, cell line 1 was significantly different (less up-regulated) compared to cell lines 2 ($p < 0.0001$), 4 ($p = 0.0087$) and 6 ($p = 0.013$); cell line 2 was significantly different from cell line 3 ($p = 0.0037$). Thus, spheroids from some cell lines require IL-1 β stimulation to up-regulate IL-10; however, once up-regulated, there can be higher expression levels than cell lines that have ubiquitous IL-10 expression.

Spheroid vs. monolayer with IL-1 β stimulation: When IL-1 β stimulated spheroids and monolayer cells were compared, both cell line and culture type were significant ($p < 0.0001$), with significant interaction between them ($p = 0.0003$). For monolayer cell lines that expressed IL-10 with IL-1 β stimulation, horse 4 had significantly increased expression compared to the other horses (vs. 1 $p = 0.0001$, vs. 2 $p = 0.0028$, vs. 3 $p < 0.0001$), and cell line 2 was significantly different than cell line 3 ($p = 0.029$).

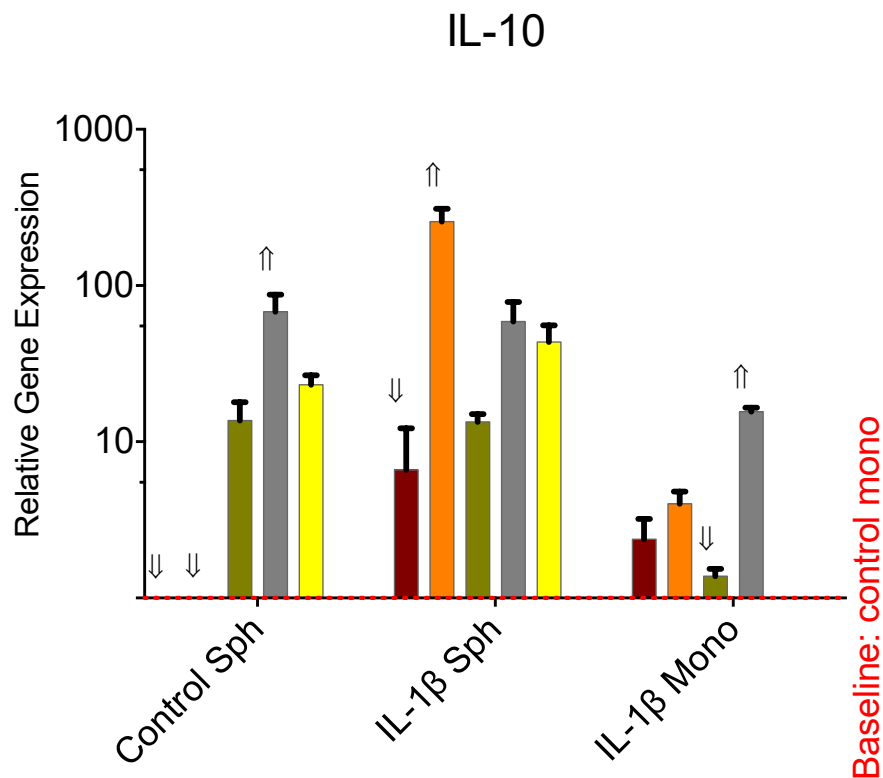


Figure 5.3 – IL-10 by cell line

Relative gene expression of IL-10 compared to monolayer cells non-stimulated with IL-1 β (monolayer control). Note that cell line 6 is compared to an average of all monolayer control cells and cell lines from horse 1 and 2 in non-stimulated spheroid culture are arbitrarily at their equivalent monolayer control baseline. Arrows, $\uparrow$ indicate significant differences and highly expressing cell lines, $\downarrow$ indicate significant differences and low expressing cell lines, per treatment group. Refer to the text for significant differences between cell lines with intermediate expression ($p \leq 0.05$). ■ Horse/ cell line 1; ■ Horse/ cell line 2; ■ Horse/ cell line 3; ■ Horse/ cell line 4; ■ Horse/ cell line 5; ■ Horse/ cell line 6.

Discussion

The aim of this study was to investigate differences in expression of key marker genes of an anti-inflammatory phenotype between spheroids from different eBMSC cell lines whether under inflammatory stimulation or not. We demonstrated that the cell donor has a significant contribution to the variation in gene expression of all the genes we investigated. Additionally, in all except IL-6, there was significant interaction between cell line and IL-1 β treatment, suggesting that individual variation results in cell lines responding differently in terms of up- or down-regulation as well as responding to different degrees of inflammatory stimulation. This finding likely explains the variation in individual response with stem cell therapy, and is of critical importance to the development of efficacious biologic therapies. Knowledge of gene responses *in vitro* prior to *in vivo* studies would allow the field to determine the most important characteristics that stem cells need to have a desired therapeutic effect.

This study also aimed to determine if spheroid culture made a difference to how eBMSCs from different cell lines responded to IL-1 β stimulation, as would be the case in osteoarthritis or synovitis. Spheroid culture impacted gene expression of all genes, but the degree or direction of change was dependent on the individual eBMSC donor. Some donor cell lines had an opposite response to IL-1 β stimulation whether cultured as spheroid or monolayer. The change from horse 5 having the least up-regulation of IL-6 in spheroid culture but the highest in monolayer culture highlights how culture type can differentially affect gene expression depending on cell line. This has clinical implications because if a patient receiving monolayer cultured cells is unresponsive, there may be an improved response with spheroid culture, or vice versa. For research purposes these findings are a reminder of how BMSCs can alter their behavior when intended, or unintended, alterations in culture conditions occur.

A biologically important finding was that different cell lines had different profiles of gene expression for the genes studied. For example, horse 6 had high levels of up-regulation compared to other cell lines for almost all genes and under almost all conditions, in contrast, horse 3 had relatively high expression of IL-6 and TSG-6 with expression of IL-10 in spheroid culture regardless of inflammatory state. However, it also had relatively low levels of mPGES-1 and PTGES2. Spheroid studies using human BMSCs have found that high levels of secretion

and expression of IL-6 and TSG-6, along with PGE₂ are an important phenotype for predicting the ability of changing macrophages from an inflammatory phenotype that produces high levels of TNF α , to an anti-inflammatory phenotype that has low TNF α and high IL-10 production [2-5,646]. However, studies of individual cell lines, and clonal populations of the given cell lines, have found that IL-10 expression is variable in human BMSCs, not only between cell lines, but within a single donor for clonal cell lines [561], and there are similar findings for IL-1ra [562]. IL-10 is directly anti-inflammatory and anti-catabolic in OA as it blocks TNF α mediated chondrocyte apoptosis and extracellular matrix breakdown [708,709]. Additionally, IL-10 can act similarly to PGE₂ to elicit anti-inflammatory effects on immune cells via a negative feedback loop [556]. Although PGE₂ is the BMSC-produced mediator found to be integral in reducing an anti-inflammatory response via alteration of macrophage response *in vitro* and *in vivo* [560,669], it is also a key sensitizer for pain during joint inflammation in murine models [710], and is found at high levels in equine models of OA [27]. As a result, a spheroid cell line with stable IL-10 expression, but reduced PGE synthases could be a good candidate for treatment of joint disease. Additionally, levels of inflammation vary within and between OA and synovitis, so spheroid culture has enhanced therapeutic potential because expression of anti-inflammatory cytokines like IL-10 can be up-regulated without requiring high inflammatory stimulation.

The method that undetected qPCR data for expression of IL-10 was analyzed could be a limitation to the integrity of these findings. However, data was not excluded or replaced with an arbitrary Ct value because those techniques have been found to lead to significant bias, and it is suggested that biologically sensible solutions may maintain generally accurate interpretation of results [711]. By using the mean of all monolayer control samples to calculate fold expression changes for cell line 6, we maintained a reference point that was within the biological scope of our data. Additionally, using reference values when no expression was detected for control spheroids would not have affected overall findings because the other cell lines were more than 10 times up-regulated compared to baseline. To support these qPCR findings, ELISA analysis of secreted IL-10 could be performed on media from all samples.

Monolayer or spheroid culture determined the response of eBMSCs to IL-1 β stimulation for all genes tested. A significant benefit of spheroid culture found in Chapter 3, as well as studies

using human BMSC spheroids [4], is that spheroids are “self-activated” to produce immunomodulatory cytokines. Additionally, spheroids were able to respond with increased vigor to IL-1 β stimulation. This was illustrated by the response of particular cell lines like horse 6 eBMSCs, which had one of the lowest expressions of TSG-6, IL-6 and mPGES-1 in monolayer culture stimulated with IL-1 β , but one of the highest when in spheroid culture stimulated with IL-1 β . Similar findings of apparent “synergy” between spheroid culture and inflammatory stimulation were found in Chapter 3 when eBMSC spheroids were stimulated with LPS. Self-activation is a key characteristic of spheroids that could increase therapeutic potential for equine OA and synovitis. Previous studies have not shown overtly positive treatment effects. For example, in a CSU osteochondral chip model of OA eBMSCs reduced PGE₂ levels but there were no other benefits [447]. Another study showed mild reduction in total nucleated cell count at increased levels of synovial inflammation treated with umbilical cord derived equine MSCs, but lameness increased [455]. We expect that the self-activation of spheroids could allow eBMSCs to have a therapeutic effect at low levels of inflammation, such as encountered in the CSU study and naturally occurring OA. At these low levels of inflammation there may not be enough inflammatory stimulation to induce significant changes in the secretome of monolayer cells. For example, 10ng/mL IL-1 β used in this study and others [315,421,707] is about 600-700 times higher than IL-1 β levels measured from human knees within 48 hours of anterior cruciate ligament injury (15.323 ± 6.9175 pg/mL) [175]. This is a reason to investigate anti-inflammatory MSC therapies at higher levels of inflammation before lower levels of inflammation. If not, inaccurate inferences about anti-inflammatory potential could be made.

To our knowledge, this is the first study to investigate differences between individual equine BMSC cell lines in response to inflammatory stimulation. The effect of inflammatory environments containing differing levels of IFN γ and TNF α , or allogeneic serum on the expression of COX-1, iNOS, IDO and IL-6 as well as expression of major histocompatibility complex (MHC) molecules from eBMSCs has been performed [712]; however, between cell line comparisons were not performed. The study did highlight that MHC-II could be up-regulated by *in vitro* stimulation with recombinant cytokines, but not with allogeneic inflammatory synovial fluid, suggesting that the levels of inflammation that eBMSCs will experience within the joint is insufficient for causing MHC-II up-regulation. MHC-II expression is a concern because

allogeneic eBMSCs with IFN γ mediated MHC-II up-regulation that have a haplotype different from the recipient haplotype can cause T cell proliferation *in vitro* [569]. A limitation of this study is that alterations in MHC expression with inflammatory stimulation, or between cell lines was not investigated, therefore potential immunologic reaction is difficult to predict from the genes assayed. However, *in vivo* work using allogeneic eBMSCs with known or unknown expression of MHCI and MHCII, and unknown haplotypes of the donor and recipient, have not shown adverse immunologic reactions after a single injection[538,564,713]. These findings remain after either intra-articular [564], or intravenous [714] repeated doses. Additionally, more current *in vitro* work shows that allogeneic BMSCs with mismatched haplotype to the recipient are able to suppress PBMC proliferation in a dose dependent manner [675]. Potentially allogeneic eBMSCs are able to avoid immune detection in the recipient, or if they are detected are able to modify the immunological response directly. Both situations may be the case as allogeneic eBMSC antibodies were found in 37% of horses when given via different routes; however there were no signs of adverse events in those horses, even when multiple doses had been administered [568]. Given these findings, use of the cell lines investigated in our study in an allogeneic manner *in vivo* without MHC or haplotype analysis is not expected to cause clinically significant limitations.

Conclusion

Individual variation results in altered gene expression up-regulated by spheroid culture or IL-1 β treatment. Out of these cell lines, use of spheroids from horse 6 would maximize production of IL-6, TSG-6, mPGES-1 and mPGES-2, and give intermediate expression of IL-10. Spheroids from horse 3 or would maximize production of IL-6 and TSG-6 but limit the expression of mPGES-1 and mPGES-2, and, importantly, increased expression of IL-10. Comparisons between spheroids with different gene expression profiles are needed to ascertain if there is a clinically relevant treatment effect. However, in the absence of testing we suggest using a cell line that optimizes IL-10 and TSG-6 levels while minimizing PGE₂ levels for treatment of equine inflammatory joint disease, such as OA and synovitis.

CHAPTER 6

Three-Dimensional Culture of Equine Bone Marrow Derived Mesenchymal Stem Cells for Treatment of Equine Synovitis

Abstract

Osteoarthritis (OA) is an incurable disease affecting humans and horses alike and inflammation is integral its initiation and propagation. This study aimed to investigate the ability of equine bone marrow derived stem cell (eBMSC) derived spheroids to ameliorate experimentally induced joint inflammation (synovitis) compared to monolayer treatment or control in a bilateral lipopolysaccharide (LPS) model. Healthy horses were randomly assigned to receive intra-articular allogeneic eBMSC spheroid or monolayer injection in one randomly assigned tibiotarsal joint. LPS was administered concurrently to induce synovitis and the contralateral tibiotarsal joint was injected with LPS and diluent control. Clinicopathologic and clinical data was collected over 72-96 hours after injection and showed that spheroid eBMSCs significantly reduced synovial fluid total nucleated cell count and lameness compared to monolayer eBMSCs 4-8 hours after injection when LPS effect was greatest. Spheroid eBMSCs also elicited a significant treatment effect compared to control for synovial fluid total nucleated cell count. This is the first study to investigate spheroid MSC use in joint inflammation or for equine veterinary applications and provides ground-work for future studies.

Introduction

OA is a degenerative, progressive and incurable disease of synovial joints causing pain and wastage in horses [15,16]. It is the most common orthopedic disease of older horses, affecting 83.5% $\geq$ 15 years old, and also affects up to one third of 2- to 3-year-old Thoroughbred racehorses [17,25]. Post-traumatic OA (PT-OA) is defined by trauma to any of the joint tissues, approximately 2% of racehorses sustain joint injury each month, which often results in OA [24]. Joint trauma causes inflammation of all joint tissues leading to a peak of inflammatory cytokines including IL-1 β and TNF α within 24-48 hours of injury [173,175,696]. IL-1 β stimulates

chondrocytes and synoviocytes to produce catabolic enzymes like matrix-metalloproteinases (MMPs) that breakdown articular cartilage, increases inflammatory cytokines including IL-1 β , TNF α , IL-6 and IL-8, and induces chondrocyte apoptosis [697]. Chondrocyte apoptosis and limited ability for cartilage repair lead to permanent damage following inflammation in PT-OA [147,700,701].

Reducing initial inflammation after joint injury reduced chemokine response, synovitis score, cartilage breakdown and bone density loss in the short to mid-term [173,184]. Synovitis was correlated with injury severity and inflammation but chondrocyte viability was not, highlighting the importance of the synovium in acute inflammation [177]. Mesenchymal stem cells (MSCs) are immunomodulatory and anti-inflammatory, trophic, anti-fibrotic and chemoattractant [702]. They have disease-modifying effects for PT-OA caused by soft tissue (rabbit, goat) and osteochondral (mouse) injuries [438,572,703]. MSCs have increased efficacy when injected early after ACL transection in rabbits or after collagenase injection in mice [572,704]. MSC anti-inflammatory activity is enhanced by high IL-1 β concentrations *in vitro*, and likewise by high levels of synovial inflammation and DAMPs in serum in murine models of OA [209,705]. Given the importance of initial inflammation in progression of PT-OA, and evidence that early MSC injection is effective, modulation of inflammation could be an integral mechanism of MSCs in PT-OA.

In an equine model of PT-OA, injection of eBMSCs did not show a profound clinical benefit [447]. Recently, a study that injected umbilical cord blood derived MSCs into horse joints with LPS-induced synovitis showed that MSCs injected alone caused an inflammatory reaction, but when injected concurrently with LPS the total number of nucleated cells was decreased at 8 hours, when the response to LPS is greatest [455]. This discrepancy in response is likely because MSCs alter their phenotype in response to activated macrophages [213,560], T cells [715] or high levels of inflammatory mediators [209], which has been labeled “cytokine licensing” [536]. MSCs that have been cytokine licensed produce soluble mediators that include PGE₂, TSG-6 and IL-6 [214,553-555]. Additionally, certain individual cell lines can produce IL-10 and IL-1ra [561,562].

Traditionally, MSCs are cultured in two-dimensional monolayer on an adherent plastic surface. However, three-dimensional spheroid culture of human BMSCs has been shown to stimulate an anti-inflammatory phenotype that expresses stable levels of PGE₂, TSG-6 and IL-6, and is capable of modulating murine peritonitis and inducing activated macrophages to become anti-inflammatory and produce IL-10 [3,4,646]. Results from Chapters 3 and 5 showed that eBMSC spheroids have an anti-inflammatory phenotype regardless of stimulation with LPS or IL-1 β . Although eBMSC monolayer cells achieve this phenotype over time in response to LPS or IL-1 β , spheroids have an increased response. Additionally, these studies showed individual variation in eBMSC anti-inflammatory phenotype, with some individuals producing IL-10 in spheroid form irrespective of IL-1 β exposure. LPS injection causes transient synovitis, with an increase in inflammatory mediators and matrix metalloproteinases in synovial fluid and cartilage, which is indistinguishable from IL-1 β injection and mimics early OA [435]. In the equine LPS induced synovitis study by [455], there was an anti-inflammatory effect of MSCs when the response to LPS peaked at 8 hours, then there were no further anti-inflammatory effects. Palmer and Bertone [436] demonstrated that the effect of LPS on carpal synovitis begins to wane by 24 hours, and is absent at 48 hours. Reduction in the inflammatory environment could have decreased the “cytokine licensing” effect of the MSCs and reduced their ability to modulate inflammation.

eBMSC spheroids have a phenotype that is “cytokine licensed” independent of inflammatory stimulation. Therefore, the objectives of this study were to determine if spheroids are effective at decreasing clinical signs associated with LPS-induced synovitis and compare the response to monolayer eBMSC treatment. We hypothesized that allogeneic spheroid cultured eBMSCs would reduce clinical signs and synovial fluid parameters of synovitis compared to untreated controls, and that allogeneic spheroid treatment will be superior to allogeneic monolayer treatment in the degree of reduction of clinical signs.

Materials and methods

Stem cell collection, characterization and culture:

An eBMSC cell line previously prepared from bone marrow aspirate from a 2-year old male thoroughbred horse with IACUC approval, was characterized and cryopreserved in liquid nitrogen vapor. eBMSCs had been determined to undergo tri-lineage differentiation, and to be Oct-4 and CD90 high (stem cell markers) and MHCII negative by flow cytometry. Spheroids from this cell line had up-regulated gene expression for PGE₂ synthase, IL-6, TSG-6 and IL-10 as described in Chapter 5 (Horse 3). Compared to 5 other cell lines cultured as spheroids, there were high levels of TSG-6, IL-6 and IL-10 but lower levels of mPGES-1 and PTGES2. First passage eBMSCs were expanded in monolayer incubated at 37°C, 5% CO₂, and 90% humidity, with media supplementation every 48 hours. Expansion media comprised of: low-glucose GlutaMax DMEM with 110µg/mL sodium pyruvate (Gibco), supplemented with 10% MSC FBS (Gibco) and 100U/mL sodium penicillin, 100µg/mL streptomycin sulfate (Sigma). At 70-80% confluence, cells were trypsinized, and 20x10⁶ cells per horse were used to form spheroids for injection using a high-throughput culture technique described in Chapter 4. Briefly, 20x10⁶ MSCs were plated at a concentration of 2x10⁶ cells/mL into a Nunclon Sphera T75 flask and allowed to aggregate for 72 hours, 10mL additional media was added at 48 hours of aggregation. For monolayer cultured cells, trypsinized cells were plated in adherent tissue culture treated T175 flasks at a density of 0.0066x10⁶ cells/cm², and grown to 70-80% confluence for injection of 20x10⁶ MSCs as per protocols for clinical application of eBMSCs routinely used in our clinic. For the final 72 hours of culture, media contained 10% autologous serum rather than 10% FBS for both spheroid and monolayer culture, eBMSCs for each horse were cultured in separate flasks, both spheroid and monolayer cells for injection were passage 3. Spheroids were harvested by aspiration and monolayer cells were trypsinized; eBMSCs were suspended in 4mL of autologous serum for injection. An autologous serum only control was prepared simultaneously to inject contralateral control joints.

Animals:

The study used 8 mixed breed horses (mean age ± SD, 7.25 ± 3.37 years, age range 2-12 years, mean weight ± SD, 502.75 ± 80.22 kg, weight range 432-601kg) that were healthy and free from

lameness or clinical evidence of tarsocrural osteoarthritis and synovitis (no associated lameness, normal range of motion, and no joint effusion) with IACUC approval. Horses were randomly assigned into groups for spheroid (n = 5) or monolayer (n = 3) treatment.

LPS synovitis induction:

Horses were randomly assigned to stem cell treatment type (monolayer or spheroid) and each limb assigned to treated or vehicle-only control using a random number generator in Microsoft Excel. After sedation with detomidine (0.01mg/kg IV), the tibiotarsal joint of the limb assigned to treatment was injected with 0.5ng LPS (*E. coli* 055:B5, Sigma-Aldrich) and allogeneic eBMSCs in 4mL autologous serum using a 20g 1.5 inch needle, and the contralateral tibiotarsal joint was injected in the same manner with 0.5ng LPS and 4mL autologous serum as a vehicle-only control.

Collection of clinical data:

Physical examination was performed at hour 0 (time of injection) and every 2 hours for the first 8 hours post injection, then at 24, 48 and 72 hours. Adverse events were classified as: signs of colic, non-weight bearing lameness, fever $\geq 101.5^{\circ}\text{F}$ or any other medical condition requiring medication administration or withdrawal from the study. Lameness examinations were performed at 0, 4, 8, 24, 48, 72 and 96 hours following LPS injection. Subjective grading was performed by 2 ACVS diplomats blinded to treatment type or treated limb (SHB, JGB) using the AAEP scale (0-5) and objective lameness data was collected using an inertial sensor system previously described and validated for hind limb lameness [716-718]. This system had increased sensitivity compared to subjective lameness grade assignment for experimentally induced lameness [719]. After lameness examination, joint effusion was graded by each ACVS diplomate as 0-4 (0 = no effusion, 1 = slight, 2 = mild, 3 = moderate, 4 = severe) [288], tibiotarsal joint flexion angle was measured by goniometry (Fig. 6.1), and tarsal joint circumference was measured with a measuring tape at the level of medially and laterally placed clip marks that were placed at the level of the medial and lateral malleolus, 2/3 of the way from the proximal aspect of the calcaneal tuberosity to the head of MTII or MTIV prior to injection at time 0 hours (Fig. 6.2). Goniometry measurements were taken at the first sign of pain (flinch,

hip-hike or movement away) detected by the flexor while slowly flexing the limb and was performed on the sounder limb first.



Figure 6.1 - Goniometry for tibiotarsal joint flexion angle

Arms of the goniometer were aligned with the tibia and plantar aspect of MTIII.

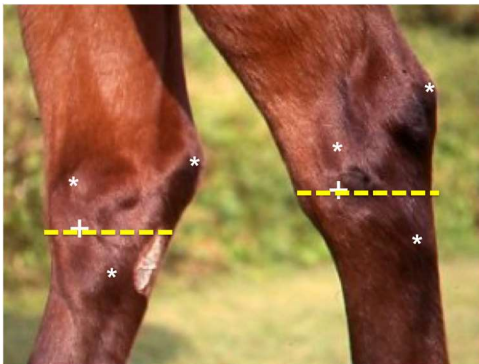


Figure 6.2 - Linear clip mark location for sequential of joint circumference measurements

Collection of blood and synovial fluid:

Peripheral blood and synovial fluid were collected at 0, 8, 24, 48 and 72 hours. Samples were immediately refrigerated and analyzed within 1 hour of collection. Complete blood counts were performed on peripheral blood samples using an automated hematology analyzer (Ac-T Diff, Beckman Coulter) and fibrinogen and total protein by spectrometry. A licensed technician at the Marion duPont Scott Equine Medical Center diagnostic laboratory performed manual synovial fluid analysis, which included manual total nucleated cell counts (TNCC) using hemocytometer, spectrometry for total protein and gross observation.

Statistical evaluation:

eBMSCs or spheroids have not been used in this equine synovitis model, therefore variance of outcome parameters was unknown and prohibited power calculations. Data was collated in

Microsoft Excel. Two-tail Student's t-tests or Fisher's exact test were performed on continuous or categorical animal data, respectively, to determine differences in age, sex, weight and left or right limb treatment between spheroid and monolayer treated groups. Inertial lameness measurements that were within the thresholds of error ($3\text{mm} < \chi < 3\text{mm}$) were reduced to 0 to represent a sound horse. The Diff Max (push off lameness) or Diff Min (impact lameness) measurements were normalized to positive measurements, and entered under the limb that was lame (treated or control). The contralateral limb was given a score of 0mm to indicate relative soundness. Data was imported into JMP® Pro 13.0.0 (SAS Institute Inc.) and continuous data was tested for normality with the Shapiro-Wilk goodness of fit test. A repeated measures 2-way ANOVA was performed to assess spheroid treated joints compared to vehicle control or monolayer treatment using a mixed model approach to account for covariate structure selected based on the lowest Akaike information criteria (AIC). Post hoc Tukey-Kramer HSD tests were performed on significant treatment effects; Dunnett's test was performed to compare differences with baseline (time = 0 hour). Ordinal categorical data was compared at each time point using Wilcoxon signed rank tests spheroid and control and Wilcoxon test between monolayer and spheroid. Statistical significance was set at $p \leq 0.05$. Data is expressed as mean $\pm$ S.E., unless otherwise noted.

Results

Animals

There were no significant differences between monolayer or spheroid treatment groups for proportion of mares/geldings ($p = 0.46$), age ($p = 0.54$), weight ($p = 0.35$) or if the left or right hind were selected for treatment with MSCs ($p = 0.46$). All horses completed the study and administration of anti-inflammatory medication (NSAIDs) was not needed. Adverse events occurred at 4, 6 and 8 hours and consisted of a fever in 1 spheroid treated horse at 6 hours (102.3°F) and 8 hours (103°F), non-weight bearing lameness in 1 monolayer treated horse in the treated limb at 4-8 hours requiring treatment after the 8 hour examination with a single dose of 0.03mg/kg intra-muscular butorphanol, and self-resolving transient agitation in one monolayer horse at 6 and 8 hours. There were no significant differences between the proportions of adverse

events in the spheroid group vs. the monolayer group (Hour 4 $p = 0.14$, Hour 6 $p = 0.18$, Hour 8 $p = 0.18$).

Clinical Data

Physical examination: Time was a significant effect on temperature ($p < 0.0001$); however, treatment (monolayer vs. spheroid) was not. Neither time nor treatment type had a significant effect on heart rate or respiratory rate.

Peripheral Blood: Systemic WBC remained within normal limits with the exception of one horse with slight leukocytosis in the spheroid group at 8 hours (12,700cells/ μ L, upper limit normal 12,500cells/ μ L). Both treatment type ($p = 0.0054$) and time ($p < 0.0001$) were significant effects on systemic WBC. The differences between higher peripheral WBC in the spheroid group vs. the monolayer group at 8 hours ($p = 0.0096$) and 24 hours ($p = 0.049$) were significant (Fig. 6.3, A). Neither treatment nor time significantly influenced fibrinogen, which remained within normal limits during the study (Fig. 6.3, B).

Joint circumference: In the spheroid treated group joint circumference was affected by time post injection ($p = 0.0002$), but not spheroid treatment vs. vehicle only control. Joint circumference was increased from baseline (0 hour) at 24 hours ($p < 0.0001$) and 48 hours ($p = 0.0011$). Spheroid vs. monolayer treatment also did not affect joint circumference, however time was a significant effect and was increased from baseline at 24 hours ($p = 0.0042$). These results suggest that LPS was the main reason for increasing joint circumference, rather than treatment with eBMSCs and that eBMSCs did not reduce joint circumference.

Goniometry: In the spheroid treated group, spheroid treatment did not affect joint angle compared to vehicle only control, however joint angle did alter with time ($p < 0.0001$). Joint angle, based on pain tolerance, was significantly increased from baseline (0 hour) at 4, 8 and 24 hours ($p < 0.0001$), but was not significantly different by 48 hours ($p = 0.34$). Similarly, treatment with monolayer vs. spheroid did not affect joint angle, however time was a significant effect ($p < 0.0001$) with increase from baseline (0 hour) at 4 hours ($p = 0.0018$), 8 hours ($p = 0.0002$) and 24 hours ($p = 0.0003$). These results also suggest that LPS was the main reason for

increased resistance to passive joint flexion and eBMSC treatment did not significantly ameliorate this effect (Fig. 6.4).

Joint effusion: There were no significant differences between joint effusion scores given by either investigator for spheroid and their control joints or spheroid and monolayer treated joints at any time point. Subjectively, joint effusion scores increased and peaked between 4 and 24 hours, then began to lower as LPS wore off (Fig. 6.5). This pattern agrees with the trends seen for joint circumference.

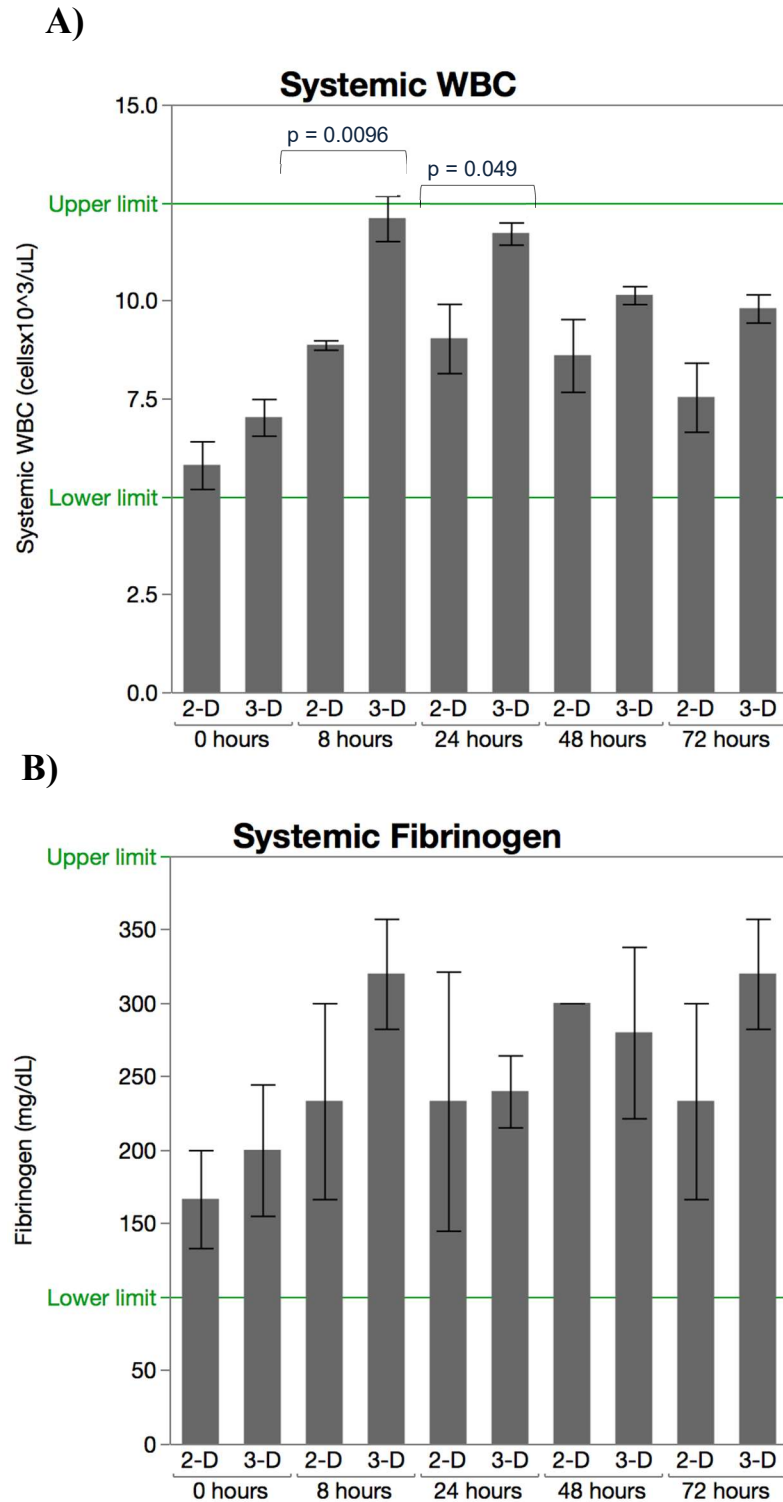


Figure 6.3 – Peripheral blood results

Graphs showing systemic white blood cell count (A) and fibrinogen (B) for spheroid (3-D) and monolayer (2-D) treated horses. The green lines show upper and lower limits of the normal range for equine values. Significant differences between data sets are shown, ($p \leq 0.05$).

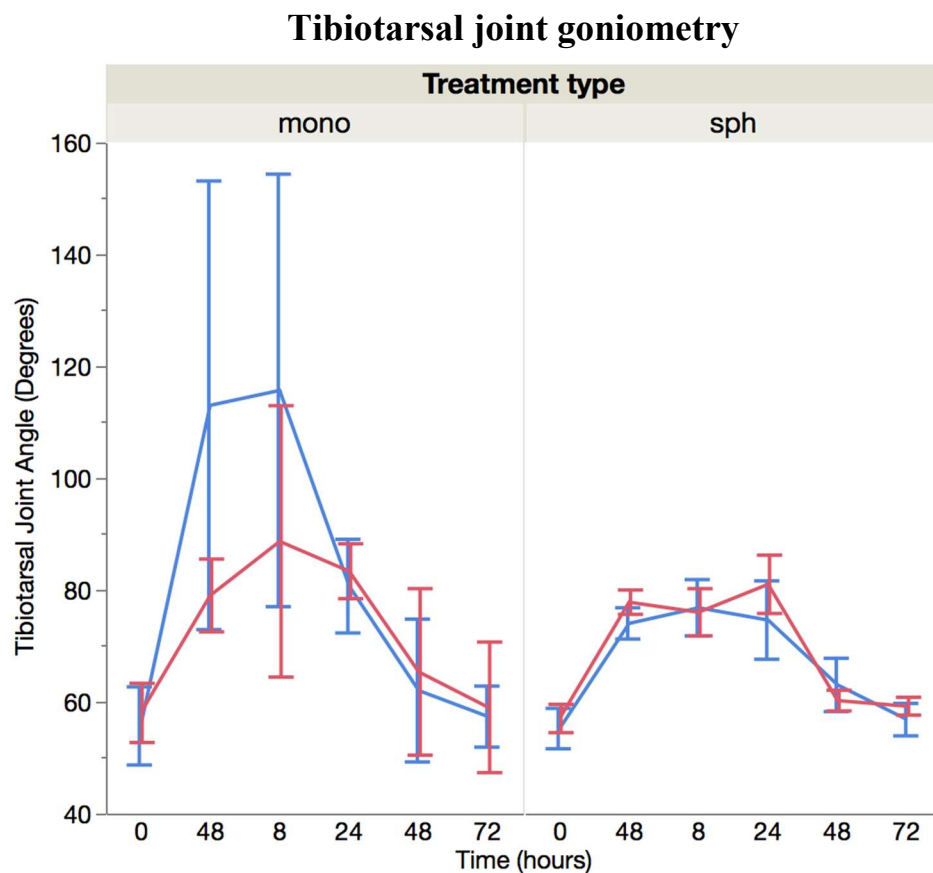


Figure 6.4 – Tibiotarsal goniometry results

Goniometry measurements by treatment type (Monolayer, Spheroid). Red line indicates treated joint angle, blue line indicates control joint angle. There were no significant differences between spheroid and monolayer treated joints, or between spheroid and their equivalent control joints.

Tibiotarsal joint effusion

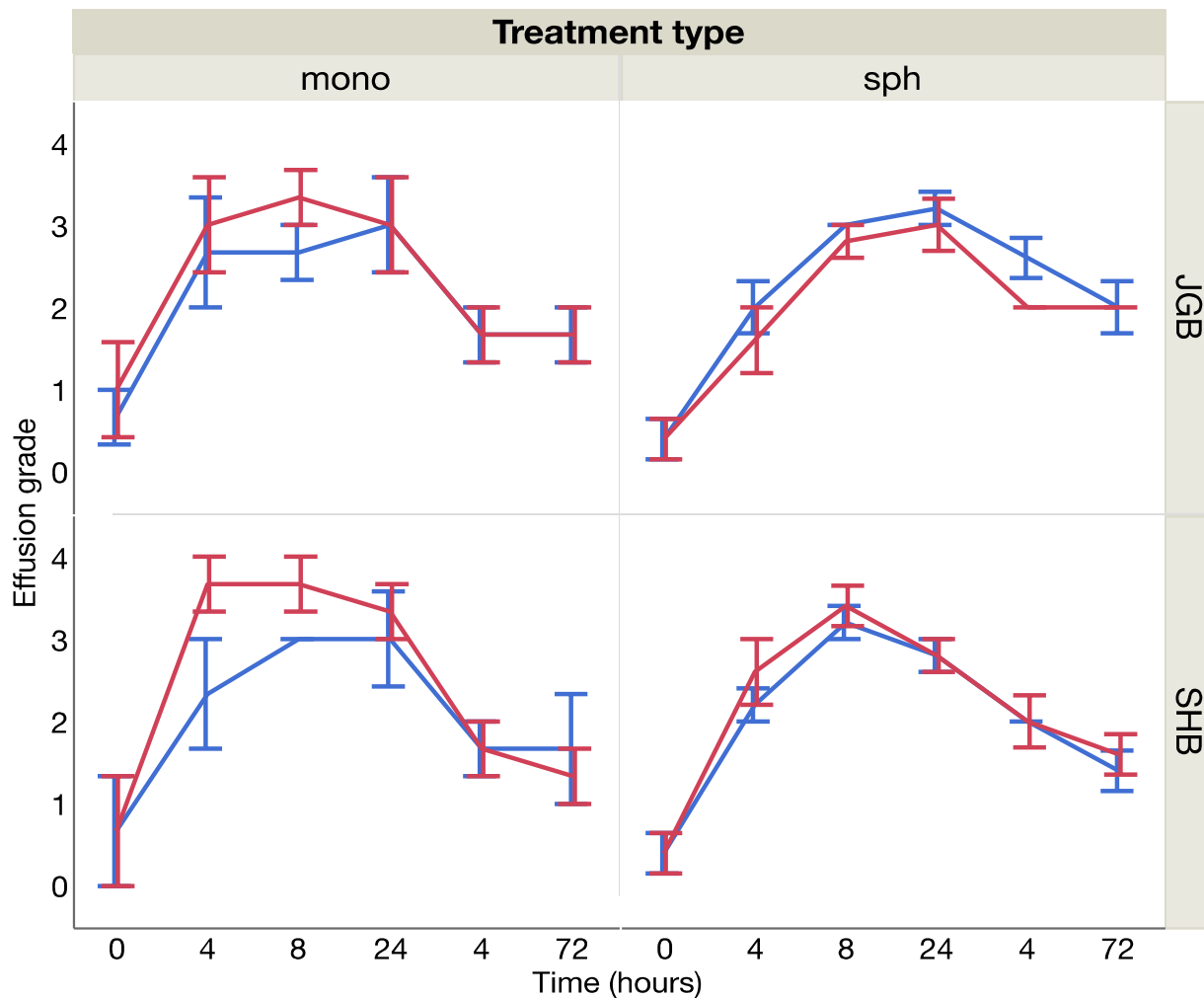


Figure 6.5 – Tibiotarsal joint effusion results

Joint effusion grades by treatment type (Monolayer, Spheroid) and investigators (JGB, SHB). Red line indicates treated joint effusion, blue line indicates control joint effusion. There were no significant differences between spheroid and monolayer treated joints, or between spheroid and their equivalent control joints.

Lameness

Subjective assessment: Subjectively, lameness was increased with LPS injection in all horses compared to baseline, which was most pronounced at 4 and 8 hours after treatment. At these times lameness at the walk was seen in all horses, from either a stiffened gait to an obvious hip hike (AAEP grade 4). By 24 hours, lameness had reduced to grade 2-3 and continued to reduce thereafter. Four of 5 horses in the spheroid group had improved lameness with treatment, and 1 of 3 horses in the monolayer group had improved lameness.

Diff Max (push off lameness): Time was a significant effect on push off lameness of MSC treated limbs ($p = 0.0030$). Inertial Diff Max measurements of treated limbs were significantly different from baseline (0 hour) at 4 hours ($p = 0.0039$). There was significant interaction between treatment type (monolayer vs. spheroid) and time ($p = 0.0112$). Post hoc pairwise comparisons showed a significant difference between lameness of monolayer vs. spheroid treated limbs at 4 hours ($p = 0.0070$).

Diff Min (impact lameness): Time point was a significant effect on impact lameness of MSC treated limbs ($p = 0.0027$). Inertial Diff Min measurements of treated limbs were significantly increased above baseline (0 hours) at 4 hours ($p = 0.0024$). There was a significant interaction between treatment type (monolayer vs. spheroid) and time ($p = 0.049$). Post hoc pairwise comparisons showed that monolayer and spheroid treatment were significantly different at 4 hours ($p = 0.018$),

Taken together, these results suggest that compared to monolayer, spheroid treatment can reduce lameness, however this is dependent on time after LPS injection and is only significant at 4 hours when LPS induced push off and impact lameness peak (Fig. 6.6).

Synovial fluid analysis

Total nucleated cell count (TNCC): In the spheroid treated group TNCC was significantly affected by time ($p < 0.0001$) and there was a significant interaction between treatment (spheroid vs. control) and time ($p = 0.035$). Post hoc pairwise comparisons showed significant difference between TNCC in spheroid treated vs. control joints at 8 hours ($p = 0.019$). There was an interaction between treatment with monolayer vs. spheroid and time ($p = 0.0025$) and time was a

significant effect on TNCC ($p < 0.0001$). Monolayer treatment was significantly different from spheroid treatment at 8 hours ($p = 0.0085$) (Fig. 6.6).

Total protein: Time was a significant effect on total protein ($p < 0.0001$) however spheroid vs. vehicle only control or spheroid vs. monolayer did not affect total protein (Fig. 6.7). Total protein was significantly increased above baseline at all time points ($p < 0.0001$).

These results suggest that spheroid treatment has the ability to reduce TNCC in response to LPS induced synovitis, however this reduction is only significant when TNCC counts peak at 8 hours. TNCC is reduced by spheroid and spheroids can reduce TNCC effectively compared to monolayer cultured MSCs. MSC treatment has no effect on the increase in total protein observed post LPS injection.

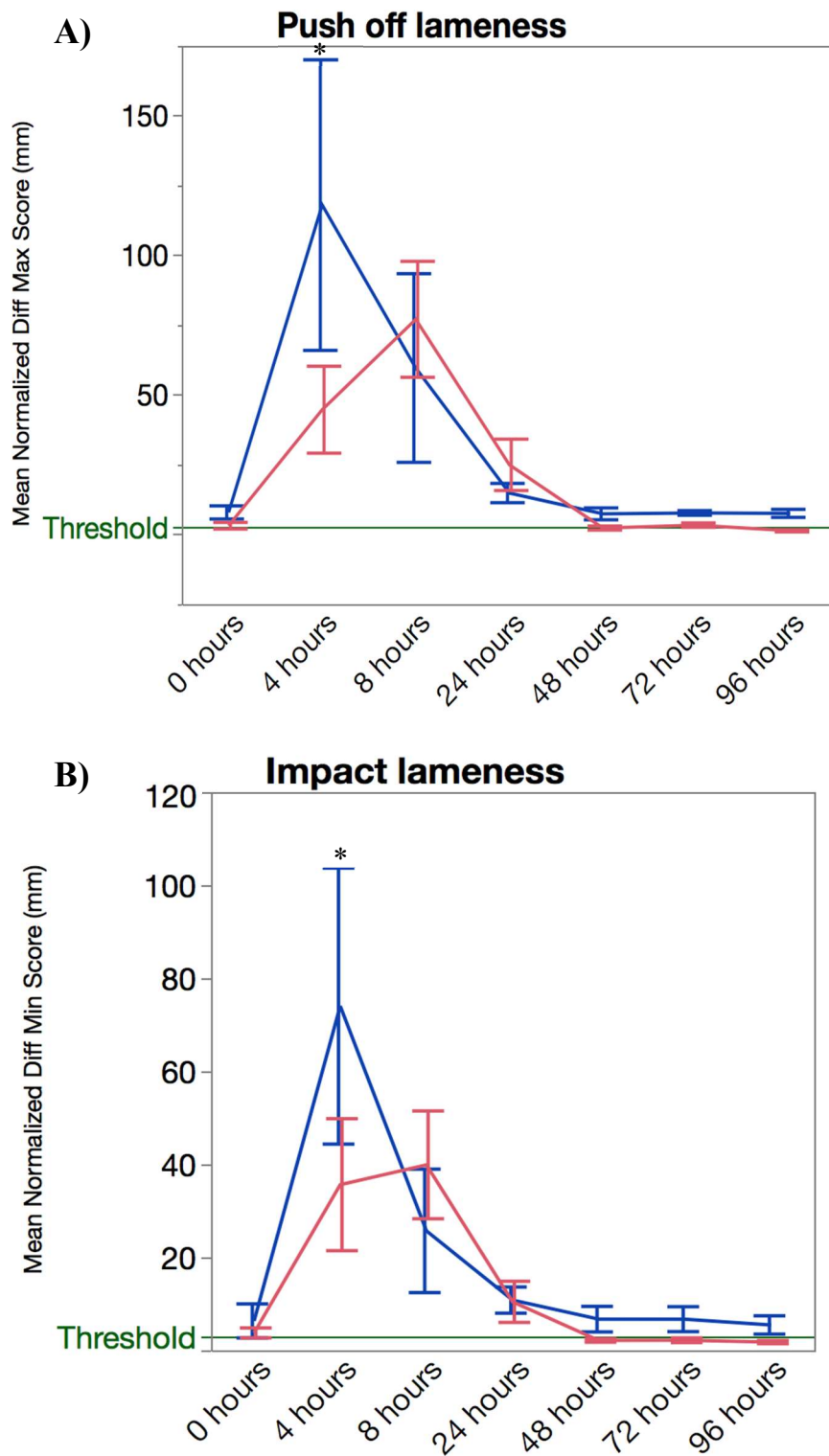


Figure 6.6 – Inertial lameness results

Graphs showing normalized inertial measurements for push off lameness (A) and impact lameness (B) for spheroid (■ red line) and monolayer (■ blue line) treated horses. Significant differences between data sets are shown by the asterisks *, ($p \leq 0.05$).

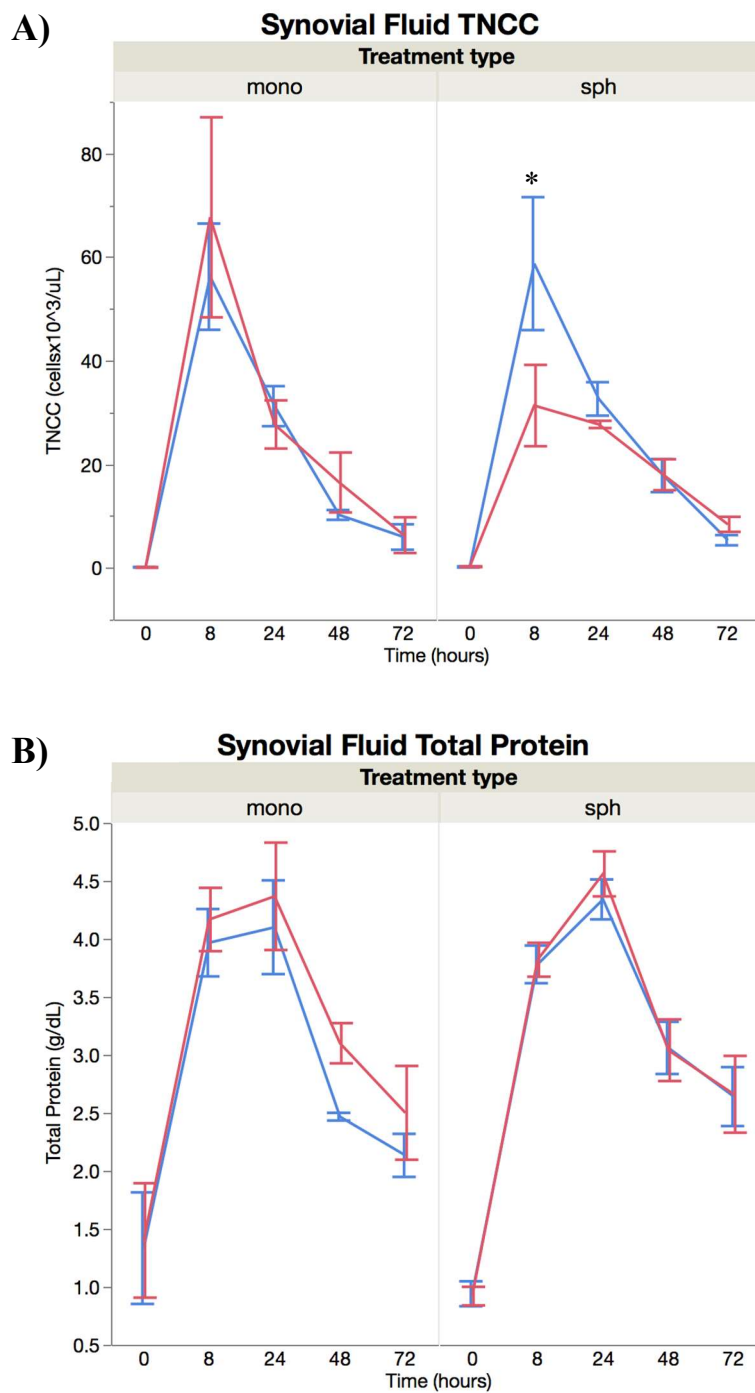


Figure 6.6 – Synovial fluid total nucleated cell count

Graphs showing synovial fluid total nucleated cell count (TNCC) (A), and synovial fluid total protein (B) in all treatment groups; ■ red line is spheroid treated joints, ■ blue line is monolayer treated joints. Asterisks * represent significant changes between treatment group and control, ($p \leq 0.05$).

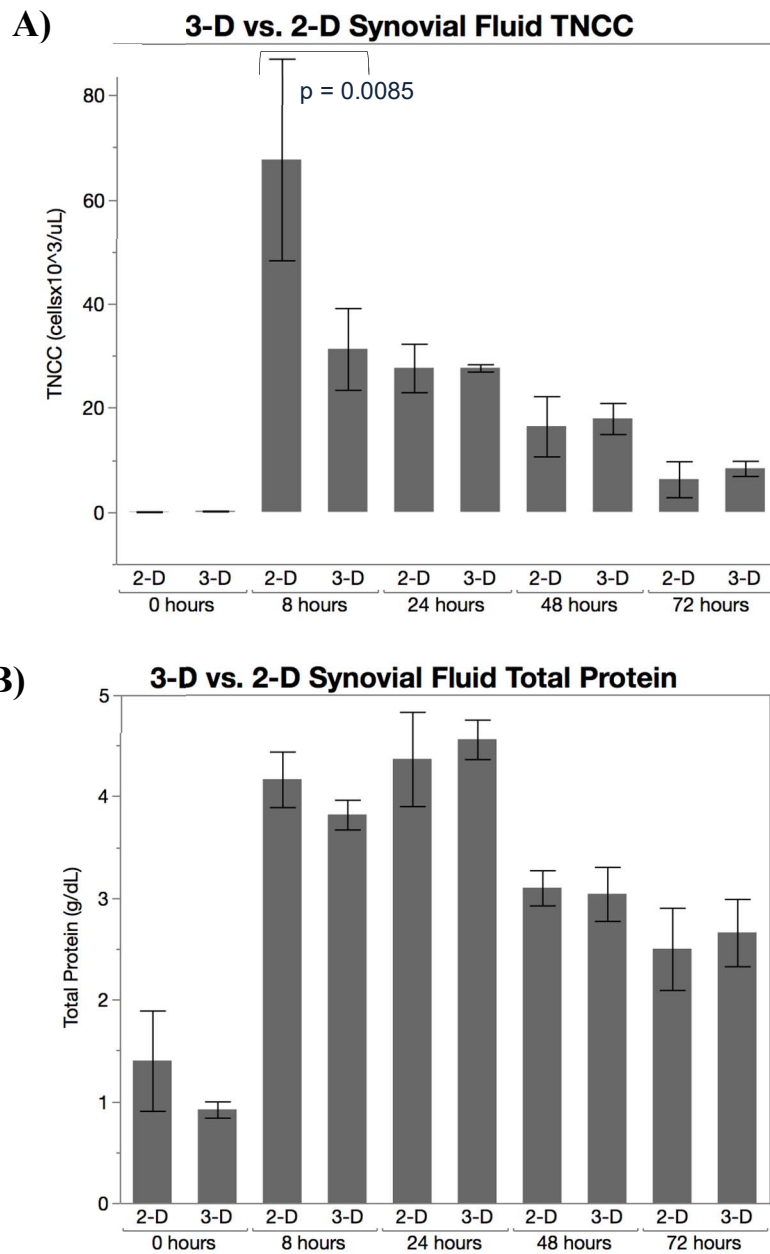


Figure 6.7 – Comparison of spheroid and monolayer TNCC

Graphs showing direct comparison of synovial fluid total nucleated cell count (TNCC) (A) and total protein (B) between monolayer (2-D) and spheroid (3-D). Significant differences between data sets are shown, ($p \leq 0.05$).

Discussion

The objectives of this study were to determine if eBMSC spheroids would be effective at reducing clinical signs associated with LPS induced synovitis. We demonstrated that inertial measurements of impact and push off lameness were significantly changed by spheroid treatment, compared to traditional monolayer eBMSC treatment, and that spheroid culture reduced synovial fluid total nucleated cell count compared to control and resulted in significantly lower cell count than in monolayer-treated joints at the time of peak LPS action. However, there were no other significant differences between spheroid and control joints, or spheroid- and monolayer-treated joints. These findings suggest that spheroids have increased therapeutic potential than monolayer cells or no treatment, but the treatment effects cannot ameliorate all biologic responses to inflammation induced by LPS. This study has allowed *in vivo* demonstration of the advantages of three-dimensional culture over two-dimensional culture to ameliorate synovial inflammation and acts as “proof of concept”, from which to base other *in vivo* studies using differing degrees of synovial inflammation or other techniques of spheroid propagation.

Treatment of LPS synovitis using umbilical cord blood derived MSCs was recently performed and found that MSC treatment increased subjective evaluation of lameness in all 6 horses compared to LPS only controls [455]. In contrast, our study showed that 4 of 5 horses in the spheroid group had reduced lameness with spheroid eBMSCs seen at 4 and 8 hours after LPS injection, which is the peak activity of LPS [436,450,451,455]. However, our small group size and the lack of sensitivity in subjective grading meant that accurate comparison of spheroid or monolayer subjective lameness scores was not possible. The AAEP grading scale lacked sensitivity in our study because lameness that is detectable at the walk is assigned grade 4. Therefore, horses with different degrees of lameness at the walk cannot be separated and horses with lameness at walk do not fit into a trot grade. Other suggestions of subjective grading scales used separately at trot and walk have been proposed [720]; however, the AAEP scale predominates subjective lameness assessment due to objective data showing fair inter-observer agreement, good intra-observer repeatability and excellence when used over time by one grader [445,721].

Use of inertial sensors is more sensitive and accurate than subjective grading for experimentally induced lameness [719]. It has good agreement with video-based motion analysis system for hind-end lameness [717] and force plate for front end lameness [444]. The data is collected as continuous measurements, divided into “push off” or “impact” depending on the phase of stride. Measurements vary in degree and direction depending on the amount of lameness and what limb is lame. This allows objective determination of gait alterations seen in the hind limbs [716]. Analysis of continuous data from inertial measurements allowed sufficient sensitivity to detect differences in lameness between spheroid and monolayer treatment groups. Complete elucidation of the effects of spheroid treatment on lameness is needed in a monolayer model of synovitis or OA so that lameness can be subjectively graded with more sensitivity.

A significant finding of this study was the ability of spheroid cultured eBMSCs to decrease the total nucleated cell count in synovial fluid at 8 hours after LPS injection. A similar study using umbilical cord blood derived MSCs also reported reduction in synovial cell counts at 8 hours; however, this reduction was mild, from approximately 120,000cells/ μ L to 100,000cells/ μ L [455]. In contrast, our study shows an approximately 50% reduction, from around 60,000cells/ μ L to 30,000cells/ μ L. Monolayer eBMSC treatment caused a non-significant rise in TNCC, and the significant difference between monolayer and spheroid TNCC suggests alteration of therapeutic potential by spheroid culture. A potential mechanism for increased efficacy of spheroids to reduce TNCC is increased secretion of immunomodulatory cytokines compared to monolayer eBMSCs. Allogeneic eBMSCs suppress proliferation of activated T-cells [675], and both horse [722] and human BMSC studies have identified PGE₂ as a key mediator [554,669]. Spheroid-cultured human BMSCs are self-activated to produce PGE₂ [2,3]. Results in Chapters 3 and 5 demonstrated increases in eBMSC spheroid culture with or without inflammatory stimulation. Additionally, Chapter 5 results demonstrated that spheroids are able to up-regulate PGE synthase, TSG-6 and IL-10 genes to a greater extent than monolayer eBMSCs in response to inflammatory stimulation. Therefore, the enhanced ability of spheroids to reduce TNCC in this study could be as a result of self-activated secretion and/or higher production of immunomodulatory mediators in response to inflammatory stimulation.

Thorough temporal biochemical and clinical investigation of LPS synovitis has shown peak inflammatory cytokine production 8 hours after injection with rapid decline beginning at 24

hours [253]. This inflammatory peak is followed by increased cartilage matrix breakdown products and matrix metalloproteinases [253]. In our study, the reduction in lameness and TNCC occurred at peak levels of synovial inflammation and cytokine production (4-8 hours). Lameness and TNCC remained elevated compared to baseline at 24 hours and during this reduced inflammatory state, there was not a significant spheroid treatment effect. Given these findings, it is unclear if the positive treatment effects of spheroids are only at high levels of inflammation in this LPS model of synovitis, or if they would ameliorate 24 hour levels of inflammation using a delayed delivery model. The lack of therapeutic efficacy after inflammatory peak could represent eBMSC need for high levels of inflammation to become activated, or that they “re-set” their threshold of immunomodulatory induction. Experiments using human BMSC spheroids to date in an anti-inflammatory capacity have been short-lived (24 hours or less) [2-4,646]. eBMSCs were used over a 49-day treatment course for experimentally induced OA and found minor reductions in synovial PGE₂ levels [447]. Not only may this model not have had sufficient inflammatory stimulation to activate eBMSCs, but treatment efficacy could have reduced over time through reduction in immunomodulatory state and/or migration away from the joint. *In vitro* studies on temporal effects of inflammatory activation on eBMSCs are needed to further understand the results seen in this study.

Three horses had adverse events in this study consisting of fevers, non-weight bearing lameness and self-resolving agitation. Fever is a commonly reported finding in the first 24 hours after LPS synovitis induction [436,450,723], and could be higher when bilateral models are used as it increases the total body dose higher than 0.5ng/mL, which is found to be the highest dose at which systemic signs of endotoxemia are not seen [436]. The horse with self-resolving agitation maintained normal vital parameters and appetite and could have been related to LPS administration her clinical signs occurred during the peak LPS-induced inflammation window.

One monolayer horse had non-weight bearing lameness and joint effusion, which were increased compared to the rest of the cohort. Alteration in response could have been due to factors in the injection preparation or the horse. Use of autologous serum (to the horse) as the injection vehicle is routine practice in our hospital and is reported by others [683]. However, there can be significant individual variation in autologous conditioned serum with significant increases in IL-1 β and TNF α after a period of surgical stress [462]. Although this is possible, it is unlikely as

the serum was not conditioned and was collected a week prior to the experiment under normal living conditions for these horses, and all horses had normal physical examinations and complete blood counts at the time of blood collection. Treatment-related reasons for non-weight bearing lameness could have been auto-immune reaction to the allogeneic cells, or induction of inflammation by non-cytokine licensed monolayer eBMSCs. Allogeneic cells have been used without adverse effects in models of tendonitis [571], and with repeated intravenous [565] or intra-articular injections [564]. Investigation of allo-antibody production within this horse could be performed; however, it is unlikely to elucidate the event of an immunologic adverse event because 37% of horses gain allo-antibodies without showing clinical signs [568]. Additionally, the lameness was resolved by 24 hours, indicating that it could have been acute transient inflammation mediated by the monolayer eBMSCs or as a result of individual sensitivity to LPS and/or joint effusion. This type of reaction is commonly called a “flare” in equine veterinary practice, which can occur from a variety of intra-articular medications. A larger study or clinical trial would need to be performed to investigate the safety of eBMSCs as monolayers or spheroids.

Although no adverse effects were seen, it was surprising that the systemic white blood cell count increased in spheroid treated horses. At 4 hours, one spheroid-treated horse was above the upper limit of normal 12,700 cells/ μ L. Although a previous study with multiple intravenous doses of allogeneic BMSCs did not elevate leukocytes, no short term analysis was performed [714]. Additionally, systemic blood was not analyzed for an LPS synovitis model using umbilical cord blood derived MSCs [455]. Therefore, it is unknown if this increase is a direct response to spheroid eBMSC treatment. Spheroid culture could potentially alter the expression of MHCII of BMSCs, which could cause a problem for horses with unmatched haplotypes. This has been found with inflammatory stimulation of eBMSCs [569]. However, the white blood counts were not significantly different from monolayer horses by 24 hours. As safety must be proven along with efficacy to develop allogeneic eBMSC products, *in vitro* experiments investigating MHCII alterations with spheroid culture as well as temporal analysis after spheroid injection in non-LPS treated horses is needed prior to clinical trials.

Conclusion

This study demonstrated that spheroid eBMSC treatment of LPS induced synovitis in equine tibiotarsal joints reduces synovial total nucleated cell counts and lameness compared to untreated control or monolayer eBMSC treatment. Further, the ability for spheroid-cultured eBMSCs to be clinically effective is dependent on time after LPS administration, with their greatest effects were observed at peak levels of total nucleated cell count and lameness. This study confirms that spheroids have an ameliorating effect on equine synovitis and have increased therapeutic potential over traditional monolayer culture. The results justify continued research to optimize spheroids for equine synovitis so that they can reach their full therapeutic potential as an allogeneic treatment for equine joint inflammation.

CHAPTER 7

Conclusions and Future Directions

Summary of results

Osteoarthritis (OA) remains a disease that progresses despite the use of previously recommended therapeutics, as they show little efficacy in the long term [274]. Current treatments ease disease symptoms in the short term but do little to ameliorate the cycle of low grade inflammation and tissue degradation that results in loss of joint structure and function over the long term.

Mesenchymal stem cells (MSCs) have shown promising *in vitro* potential to ameliorate synovial inflammation, as well as reduce cartilage degradation [585,666]. *In vivo* experimental models in rabbits, goats and mice have found amelioration of disease progression [438,572,703]; however this has not been the case for horses [447,455].

Three-dimensional spheroid culture for the up-regulation of anti-inflammatory effects of MSCs has been tested *in vitro* and in murine models of inflammation [3,4], but not for joint inflammation, and not in large animal models. This dissertation has reported that the anti-inflammatory properties of equine BMSCs (eBMSCs) are enhanced through three-dimensional spheroid culture, developed processing protocols to increase clinical relevance, increased understanding of allogeneic eBMSCs to make clinical application more timely, and demonstrated that spheroid-culture of MSCs may increased therapeutic potential over traditional culture methods.

This dissertation introduced eBMSC spheroid culture as a method of “cytokine licensing” without the need of exposure to exogenous cytokines or an inflammatory environment. Similar responses were found compared to human studies, with increased production of PGE₂, TSG-6, IL-6 and SDF-1 compared to monolayer culture. The spheroids are considered to be “self-activated” because, without being exposed to exogenous factors, they up-regulate immunomodulatory cytokines that have been shown to reduce inflammation *in vitro*, and *in vivo* in both humans and horses [4,553,554,669,724].

The main limitation of using spheroids instead of monolayer culture in equine clinical practice was the need for a more efficient preparation of the high numbers of cells needed for therapy. Additionally, as most eBMSCs are administered by hypodermic needle injection, there was concern for the ability to administer an enlarged aggregation of eBMSCs without inducing cell death or senescence. The second study showed that formation of eBMSC spheroids in commercially available non-adherent flasks was possible without detriment to the transcription of genes important for MSC anti-inflammatory effects. Additionally, spheroids could be injected through 22g, 20g and 18g needles commonly used in equine practice without harming viability. In fact, spheroid eBMSCs had lower senescence and increased viability compared to monolayer eBMSCs. These properties gave confidence that eBMSC spheroids could be used in clinical applications.

Ready availability of eBMSCs derived from the recipient's own tissue is not usually possible because they take 3-4 weeks to propagate; longer if the animal is aged [725]. Allogeneic eBMSCs offer a solution for readily available "off the shelf" use; however, variation in the production of IL-10 was seen in the first experiment between 3 stem cell lines. Therefore, investigation of anti-inflammatory properties of spheroids between a larger group of cell lines was an important step to optimize the anti-inflammatory properties of spheroid eBMSCs and determine individual variation in the anti-inflammatory phenotype of eBMSCs. The third study showed that there was significant variation between cell lines of eBMSC spheroids for the production of genetic markers of an anti-inflammatory phenotype; TSG-6, PGE synthases, IL-6 and IL-10. It also demonstrated variations in "profile" of anti-inflammatory markers, for example some cell lines were highly up-regulated for all genes, while others had up-regulation of TSG-6, IL-6 and IL-10 but not PGE synthases. From these results, and an understanding of the role of inflammatory cytokines in joint inflammation and pain sensitization, a stem cell line could be chosen for allogeneic spheroid application *in vivo*.

The final study culminated the *in vitro* work by testing the efficacy and safety of spheroid eBMSCs in an *in vivo* model of synovitis. This study investigated the ability of equine bone marrow derived stem cell (eBMSC) derived spheroids to ameliorate experimentally induced joint inflammation (LPS synovitis) compared to monolayer treatment or control. There was a significant reduction in objective lameness measurements and synovial fluid total nucleated cell

count in this study. The findings elicited information about the biology of eBMSC spheroids *in vivo* that warrant further investigation, such as the timing of significant effects coincided with peak levels of synovial inflammation.

This thesis tells a story of how one aspect of MSC biology can be investigated and optimized *in vitro* for enhanced benefit *in vivo*. The results of these studies demonstrate the complex interactions between MSCs and their disease environment, as well as each other. It also highlights that manipulation of MSCs through alterations in culture condition or cellular density can result in an advantageous or detrimental effect in a patient, as well as inherent variability in MSCs from different individual donors. Researchers and clinicians owe it to the field of biologic and regenerative therapies to fully understand and investigate biological processes that could impact product quality and/or therapeutic effects.

Future directions

Although this work has demonstrated spheroid culture as a pre-activation technique to increase anti-inflammatory properties, little is known about the duration of this effect, if it occurs under less inflammatory stimulation, or if there are other manipulations that could enhance spheroid anti-inflammatory activity further. Direct progression of this work could include: 1) *in vitro* models of OA stimulated with different levels of recombinant cytokines to assess inflammatory level on the ability of spheroids to ameliorate inflammation; 2) time course experiments either *in vitro* or *in vivo* over longer periods of time; or 3) similar *in vivo* experiments with delayed injection of eBMSC spheroids. Additionally, pre-treatment with exogenous cytokines or hypoxia could be investigated as methods to increase or prolong anti-inflammatory potential.

The *in vivo* portion of this dissertation highlighted significant questions about allogeneic eBMSC spheroid use, which is important to investigate prior to clinical trials. There was a significant increase in systemic white cell count in spheroid treated horses but not monolayer treated horses. Potential immunogenic effects of spheroid culture need *in vitro* investigation, such as up-regulation of MHCII with spheroid culture or inflammatory stimulation of spheroid cultures. Depending on results, further *in vivo* safety studies such as intra-dermal testing for immunogenicity could be performed.

In summary, the future direction of eBMSC spheroid research is multifaceted and promising. These suggestions are a portion of the future directions outlined in the discussions of each study. No matter what direction eBMSC spheroid research takes, strict adherence to understanding and investigating the biological effects of culture condition changes *in vitro* to increase likelihood of an idea becoming a success *in vivo* is essential.

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