The Development and Use of Canine Adipose Derived Stromal and Progenitor Cells to Treat Osteoarthritis

A Dissertation

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Dedication

To Rapha, who maintains the characteristics I desire to maintain during my career.

Abstract

We evaluated the viability of canine adipose derived stromal cells (ASC) in the presence of osteoarthritic joint fluid, determining that exposure to osteoarthritic joint fluid is more cytotoxic than exposure to normal synovial fluid. We demonstrated that diluting the joint fluid diminishes the severity of this effect. We have demonstrated that a stromal vascular fraction (SVF) preparation of ASCs is phenotypically different from cultured ASCs, having a greater expression of proinflammatory mediator (IL-1β (interleukin-1 beta), COX-2 (cyclooxygenase-2)) and anti-inflammatory mediators (IL-1ra (interleukin-1 receptor antagonist), TIMP-2 (tissue inhibitor metalloproteinase-2) mRNA levels than cultured cells, and greater variability in expression of cell surface markers (MHCI, MHCII, CD90, CD34, CD44 and CD45). We evaluated multiple types of culture media, and found that there is some variation in the previous mentioned markers and mediators, but not a significant difference. Consistent tri-lineage differentiation of ASCs appeared to differ amongst different media types. We concluded that media should be selected according to a phenotypic profile that would be beneficial for the disease the ASC therapy is targeting. We assessed in vivo safety and efficacy of canine autologous SVF and allogeneic ASC therapy in dogs diagnosed with osteoarthritis secondary to a medial coronoid process in the elbow, utilizing objective outcome measures, including ground reaction forces (GRF) and delayed gadolinium enhanced magnetic resonance imaging of cartilage (dGEMRIC). We found no significant deleterious side effects with either therapy, and have produced support for the use of allogeneic ASC therapy.

Table of Contents

List of Figures

Chapter 1

Introduction

Use of Stem Cell Therapy in Canine Veterinary Medicine

Stem cell therapy has become a focus of great interest across all species for multiple therapies. The use of stem cell therapy in dogs has the potential to aid veterinary medicine for improved patient health, quality of life, and longevity of life. It also provides preliminary data for humans in many disease processes as a large animal model. There are many naturally occurring disease processes in the dog that reflect good disease models for evaluating the safety and efficacy of novel therapeutics. Thus, investigation of stem cell therapy in canine naturally occurring and induced disease processes has become extremely prevalent in the last decade. Regulations that exist in human medicine are not always applicable in veterinary medicine, often allowing the administration of therapeutics that are not properly investigated, stringently regulated, or efficacious. There has been financial incentive within the veterinary industry, often driven by owners themselves, to utilize stem cell therapy. Unfortunately, it may be that this financial investment is unwarranted. Stem cell therapy requires much more investigation to determine source characteristics, ideal administration, and cell behavior in normal and diseased biologic environments. A Pubmed search at the time of this thesis writing produces 1578 articles under the search criteria “canine stem cells”, and yet most of these publications raise more questions than answers. This thesis will be focusing on stem cell therapy and biology as it applies to clinically relevant problems.

In general veterinary practice, stem cells are currently limited to one of two mesenchymal

stem cell sources. Clinically available stem cells are derived from bone marrow aspiration or adipose tissue harvest. [1-8] Most preparations in use include a minimally processed product that is generated from the bone marrow or adipose stroma. [2,9,10] Bone marrow derived mesenchymal stem cells (BMSC) have been under investigation longer and have gained a strong presence in equine clinical veterinary medicine, while being minimally utilized in canine clinical practice. [11-14] Either source in dogs generally requires general anesthesia, whereas BMSCs can be obtained from a standing, sedated horse. ASCs may be more difficult for the equine veterinarian to obtain under the same plane of sedation, and would be more likely to leave a visible scar, which may be less desirable and acceptable in the equestrian world. Aside from convenience, behavior and disease process should be a consideration, but does not always receive the attention it should. The effects, phenotype, differentiation capacity, and behavior of mesenchymal stem cells are not equivalent between sources. [2,3,6-8,11,15-17] Equine veterinarians are faced with a greater number of ligamentous and tendinous injuries than the small animal veterinarian, likely due to the activity and expectations of horses. BMSCs have been investigated much more thoroughly in equine medicine, and appear to be efficacious in these injuries. [12-14] ASCs are generally accepted to have a greater anti-inflammatory capacity than BMSCs, and thus have a high clinical application in canine medicine. [16-18] The primary clinical focus in canine veterinary medicine has been osteoarthritis, although practitioners certainly do not limit their imaginations or practices to this disease, in spite of a distinct lack of published data to support such practice. [9,10,19,20] The pathology of arthritis involves an ongoing inflammatory process, hence the appeal for an "anti-

inflammatory" capable product. [21,22] Research investigations have investigated many other sources of stem cells, including induced pluripotent stem cells (iPS), umbilical cord derived stem cells, embryonic stem cells, as well as almost any other adult tissue. [23-30] While these provide excellent means to investigate multiple disease processes, these cells are not readily available to the general practitioner.

There are multiple publications in canine veterinary medicine assessing a variety of disease processes and their response to stem cells. Much of what has been investigated either has a more academic application, or may be self-limiting because they include implants, devices or equipment not accessible to the practitioner. For example, intervertebral disc disease shows great potential to benefit from stem cell therapy, either by targeting the spinal cord or the disc itself, but is not a disease conducive for direct administration of cells in general practice. [31-33] Many of these diseases prompt study and investment because of their application to human medicine, so are not necessarily designed to translate into the veterinary general practice. In the next several years we will likely see a vast expansion of clinical therapy for a much greater variety of disease processes, but currently, osteoarthritis has some limited literature support, and remains an accessible therapeutic option for practitioners and owners alike. [9,10,19,20]

Review of Canine Osteoarthritis

An estimated 1 in 5 dogs in the United States suffer from osteoarthritis affecting elbows, knees and hips. Osteoarthritis is classified as a non-inflammatory arthritis, due to the relatively low number of involved inflammatory cells compared to those arthritides

classified as inflammatory. While this provides some clarification between highly inflammatory, typically more severe arthritic conditions, and the more insidious osteoarthritic condition, it may falsely be presumed that inflammation is not an active component of osteoarthritis. This is inaccurate, as there are many commonalities in the mediators of both classified arthritides with many of the same cytokines, prostaglandins, leukotrienes, destructive enzymes and inflammatory cells. [21,22,34,35,35-37]

There is a much greater component of biomechanical instability within osteoarthritis than rheumatoid arthritis. It is a complex interaction between biomechanically destructive forces, cartilage damage, and secondary inflammatory mediators that causes cartilage destruction and joint inflammation. The inciting agent for this disease process is typically traumatic, either abnormal forces on a normal joint, or normal forces on an abnormal joint, resulting in a much slower progression than inflammatory arthritides. This elicits a cascade of events that leads to disruption of articular cartilage, the underlying subchondral bone and normal synovium physiology. The precise mechanism by which this occurs is still not clearly defined. [22]

Once cartilage is destroyed or damaged, the disease is non-reversible. Through the complex pathway that is triggered, the disease tends to progress over the life of the animal, resulting in clinical symptoms over many years that limit patient function and quality of life. [22]

Because we have yet to achieve cure of this disease process, there are many therapeutic options available, all of which have limitations and variable efficacy. In some occasions, an underlying orthopedic disease process may be surgically corrected or addressed in an

effort to minimize osteoarthritis progression. [38-44] As the arthritis already present is non-reversible, this still often results in patients needing additional intervention as their life progresses.

A major focus of pharmaceutical management of osteoarthritis in dogs are non-steroidal anti-inflammatory drugs (NSAIDS). There are multiple products on the market, available for prescription that have demonstrated efficacy in clinical outcome. [45-58] While highly efficacious, and typically very safe, these patients often require life-long therapy or may have concurrent disease processes, which may lead to deleterious side effects. [45-47,50,54,56,59-62] There is much focus on the use of cyclooxygenase-2 (COX-2) selective NSAIDS, which target a major inflammatory cytokine in the disease process, while sparing other multi-functional cytokines that play roles in homeostasis, and contribute to deleterious side effects noted with NSAID use. [59,60,63] NSAIDS may not be a feasible option for all patients.

Weight loss has been demonstrated to be an efficacious means of reducing clinical symptoms of osteoarthritis, and often is combined with physical therapy to maximize efficacy of medical management. [64-66] While proven efficacious, this can be challenging to owners, financially, emotionally and in regards to time commitment. There is often a struggle with compliance utilizing this modality.

Nutraceuticals have gained a lot of attention and are widely used as therapies for osteoarthritis. The data to support them is variable, often involving poorly designed studies, or producing conflicting results. The two nutraceuticals that have gained the most attention and have the widest use include glucosamine chondroitin and omega-3

fatty acids. Glucosamine and chondroitin are both constituents of cartilage, and administration of supplementations are thought to potentially replace these factors that are lost during osteoarthritis degradation. Results of clinical trials assessing them have been very conflicted in outcome. [67,68] It appears to have very minimal deleterious side effects, but can generate an added financial burden that may not provide benefit for the pet. There is better evidence to support the use of omega-3 fatty acids, for the anti-inflammatory effects against osteoarthritis, and minimal deleterious side effects are noted. There is also potential to reduce the need for NSAID therapy when supplemented with omega-3 fatty acid supplementation. [69-74]

As molecular technology and genetic therapies have advanced, there has been increasing interest in modalities targeting specific mediators associated with minimizing clinical symptoms of osteoarthritis. Tissue inhibitor metalloproteinase 2 (TIMP-2) has been demonstrated in multiple studies to be an important factor for maintaining a healthy joint environment, and is often decreased in osteoarthritic joints. Multiple metalloproteinases have been identified and correlated to destruction of the joint environment and are targeted by TIMP-2. [21,36,75-80] Interleukin 1 beta (IL-1β) has been identified as a potent proinflammatory mediator in osteoarthritis, and it's antagonist, interleukin 1 receptor antagonist (IL-1ra) has shown great promise in alleviating much of the deleterious effects of IL-1β. [21,76,81-83] While these therapies show promise, they are not readily available for the practitioner, and remain possible future therapeutics.

Due to the limited options and progressive nature of the disease process, owners, veterinarians and investigators remain keen to identify alternative methods of therapy,

which has likely led to the massive adoption of stem cell therapy for osteoarthritis.

Use of Stem Cells in Osteoarthritis in Medicine

Stem cells as a therapy for osteoarthritis have two theorized potential mechanisms of action. Initial interest was in the differentiation capacity of stem cells, in hopes that the damaged cartilage could be replaced with new, healthy chondrocytes. This is a logical pursuit, as mesenchymal cells are vital for healthy tissue homeostasis and cell replacement in the adult. [84] Mesenchymal stem cells have shown the capacity to engraft and differentiate within host tissue in multiple species and locations, but to date have poor demonstration of engraftment within joint cartilage without aid of an implant. [85-92] There are a few reasons why direct implantation without structural support may not allow or support cartilage regeneration. The osteoarthritic joint is an inflamed structure, with local cell and stromal destruction. [22] This may endanger cell viability and prevent engraftment. The cartilage stroma has a relatively low vascular content, and thus cells may not have the physiologic or metabolic support to engraft and survive. [93,94] Finally, the joint is a mobile and a fluidic environment, which allows consistent motion across damaged surfaces and disruption of cells transplanted. While cartilage resurfacing is a valuable and excellent pursuit, it appears to require bioengineering capabilities, and is problematic when large defects are present. There is still much to be learned and studied in this application of stem cell therapy.

The current modalities of therapy utilized in practice are taking advantage of the immunomodulatory capacity and trophic effects that mesenchymal stromal cells (MSC)

provide. A portion of these MSC preparations contain a small stem cell population. [16-18,95,96] ASCs in particular have a remarkable capacity to immunomodulate their environment, making them very attractive as a therapy for active inflammatory conditions. [9-11,16-20,95,97-99] MSCs have been investigated for therapy of common arthrides in many other species, and osteoarthritis has received particular attention in canines and equines. [9,10,19,20,100-10511,106] In addition to reducing inflammation and inflammatory responses, MSCs have the capacity to recruit local host cells inducing a response that may not otherwise be present. [95,97,101,104,107] While these mechanisms of action are not specifically targeting cartilage regeneration, reduction of the inflammatory response is likely to be chondroprotective against these progressive disease processes.

The capacity of MSCs to be immunomodulatory is significant enough that allogeneic MSCs are better tolerated than other grafts, being accepted and incorporated into the local environment. Allogeneic MSCs have been widely investigated for other disease processes, but neglected in osteoarthritis in canine medicine. [32,108,109]

The option for osteoarthritis therapy has become widely available to the general practitioner, and owner motivation has driven wide use in the clinic with a poor understanding of the best means of therapy. Most practitioners administer the cells intra-articularly, at an arbitrary dose, timing and frequency. [9,10,19,20] Comparison of dosage schemes, site of administration, and effect of redosage has been ignored in investigative trials. Regulation and characterization of product is very poor in veterinary medicine, with widely variable products available on the market, making direct extrapolation from literature inappropriate if protocol is not similar. Until preparation of this manuscript,

objective data validating their use has been lacking to the best of the author's knowledge.[9,10,19] A very recent publication provides the first look at force platform gait analysis in dogs treated for osteoarthritis with ASCs, and shows further support for their efficacy.[20] These data should be interpreted cautiously, however, as there was a small number of dogs enrolled, and the therapeutic agent was significantly different than what has been reported previously, adding platelet rich plasma to the therapy.[9,10,19,20]

A better understanding of what product is in use, the ideal application for that product, and investigation into appropriate administration should be pursued before uncontrolled use and poor understanding damages availability and reputation of this therapeutic modality.

The purpose of this work is to investigate a defined canine ASC product, and assess the best clinical application of that product. An in vitro assay evaluating the effect that osteoarthritic synovial fluid has on the viability of canine ASCs questions the current, most common protocol of direct intra-articular injection into the joint, and indicates trophic effects of ASCs may be more prominent than previously thought. Evaluation of ASCs as a SVF product (as is currently used in practice) compared to ASCs that have undergone culture expansion as expected with allogeneic ASC therapy was undertaken. We specifically evaluated markers and mediators suspected to play a role in ASC mechanisms of action or in the therapy of OA. We also evaluated multiple culture conditions to assess the variability of phenotype of cells under differing conditions. Using this information, a culture condition was selected to produce an allogeneic ASC preparation selecting for minimal proinflammatory mediator expression, and autologous

ASCs and allogeneic ASCs were compared in a clinical trial for treatment of a defined OA condition using objective outcome measures.

Chapter 2

Canine adipose derived stem cell viability following exposure to synovial fluid from osteoarthritic joints.

Introduction

The treatment of canine osteoarthritis (OA) with adipose derived stromal cells (ASC) has become quite prevalent in general practice following publication of evidence of improved clinical symptoms following treatment. [9,10,19] This treatment consists of a fat sample that is processed to allow release and collection of all nucleated cells from the stroma, and is referred to as a stromal vascular fraction (SVF). The typical means of administration is an intra-articular injection of the SVF. This allows direct application to the area of disease. An alternative means of administration would be intravenous injection, however this method is less used, as studies have demonstrated that the majority of intravenously administered stem cells are filtered out by the lungs, liver and other peripheral organs. [110-112] The safety and efficacy of each means of administration has not been compared in dogs.

The osteoarthritic joint is an unfavorable environment for local cellular health and viability. There are many mediators that promote inflammation, destroy cartilage, or induce apoptosis. [113-117] The effect of this environment on local cells has been investigated via in vivo and in vitro experiments, and found to be detrimental to the health and viability of synoviocytes and chondrocytes. [113,117-120] This raises questions regarding the viability of transplanted cells into such an environment.

The objective of this study was to investigate the viability of canine ASCs when exposed to osteoarthritic synovial fluid, and determine if dilution of osteoarthritic synovial fluid altered cell viability. We adopted a null hypothesis that exposure to synovial fluid from an osteoarthritic joint would not reduce canine ASC viability.

Materials and Methods

Owner consent was obtained and all procedures were performed in accordance with the University of Minnesota Institutional Care and Use Committee, protocol number 1006A83372.

Isolation of Adipose Derived Stromal Cells

Falciform adipose tissue was harvested at the time of surgery from five healthy dogs admitted to the University of Minnesota College of Veterinary Medicine for abdominal surgery unrelated to the study. Dogs with neoplasia or septic medical conditions were excluded. Adipose tissue was processed according to previously reported protocols for isolation of the stromal vascular fraction. {Black,L.L. 2007; Black,L.L. 2008} The nucleated cell fraction was than placed into Keratinocyte N-acetylcysteine (KNAC) cell medium for ASC expansion consisting of modified MCDB153 medium (Keratinocyte-SFM) (Gibco, Life Technologies, Grand Island, New York), supplemented with: 2mM N-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO), 0.2mM L-ascorbic acid 2-phophate (Sigma-Aldrich, St. Louis, MO), 0.09mM calcium and human recombinant epidermal growth factor (5ng/mL) (Gibco, Life Technologies, Grand Island, New York), bovine

pituitary extract (50ug/mL) (Gibco, Life Technologies, Grand Island, New York), insulin (5ug/mL) (Sigma-Aldrich, St. Louis, MO), hydrocortisone (74ng/mL) (Sigma-Aldrich, St. Louis, MO), 5% FBS (Hyclone, Thermo Fischer Scientific, Minneapolis, MN), and 1% antibiotic (Mediatech Inc, Corning, NY) at 37C in a humidified 5% CO2 atmosphere. {Kang,J.W. 2008}

Synovial Fluid Samples

Synovial fluid samples were collected from dogs with pain and lameness secondary to OA and from normal dogs with no joint disease. The presence of OA was confirmed by owner history, orthopedic exam, radiographic exam, and when surgical intervention was indicated, visual identification of OA at the time of surgery. Normal synovial fluid was harvested from dogs that were euthanized for an unrelated research study. These dogs had no history of lameness, a normal orthopedic exam, and a visually normal joint assessed following synovial fluid collection. All synovial fluid samples were centrifuged at 400g at 4C for 6 minutes. The supernatant was aspirated from the pellet to eliminate any cellular contamination of the joint fluid and stored in a -80C freezer. Volumes ranged from 0.25 ml to 0.5 ml from normal joints, and 1 ml to 3 ml from OA joints. All samples underwent three freeze-thaw cycles to further eliminate intact cellular contaminates. Synovial samples were pooled according to treatment group prior to dilution, to eliminate variability across the assay and provide enough fluid to test all cell lines in duplicate with each treatment. Due to the small sample sizes, molecular characterization was not feasible.

Cytotoxicity Assay

The ASCs from passages 2-4 of each of the five donors were plated at 10,000 cells/well in a 96 well plate in duplicate for each condition.

Once the cells were confluent (typically within 24-48 hours), each cell line was treated with each condition in duplicate: 100 uL of normal synovial fluid, 100 uL of a specified dilution of synovial fluid from OA joints, or 100 uL of medium containing no synovial fluid. Synovial fluid derived from OA joints were used as a no dilution treatment, and the following serial dilutions: 1:1 (1 part medium, 1 part synovial fluid), 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 or 1:10 dilution, utilizing KNAC growth medium as the diluent. Cells were placed in these conditions for 12 hours. The contents of each well was then aspirated and placed in a sterile centrifuge tube. The well was rinsed with phosphate buffered solution two times, and each rinse added to the aspirated well contents. Cells were then detached using Tryple-E (Invitrogen, Life Technologies, Grand Island, New York), which was inactivated by the addition of KNAC growth medium after 10-15 minutes. The contents of the well were aspirated and added to the previous well contents. The well was rinsed two more times with phosphate buffered saline, and each rinse added to previous well contents. The accumulated well contents were centrifuged at 400g at 4C for 6 minutes. The supernatant was aspirated and cells were resuspended in 500uL of KNAC growth medium.

Viability of cells were counted using the trypan blue (Invitrogen, Life Technologies, Grand Island, New York) exclusion method, with cells exposed to dye at a 1:1 dilution

for five minutes before counting. [121,122] A hemocytometer was used to count cells. The individual counting each treatment was not aware of group assignment until after counting. A percent viability is reported, and was calculated by dividing the number of viable cells (non-stained cells) by the total number of cells (stained and non-stained).

Statistical Analysis

Statistics were analyzed with the aid of StatPlus 2009 software. Viability of treatment conditions were analyzed using Wilcoxin signed-rank test, with a $p<0.05$ considered statistically significant. Data was further analyzed using a linear regression analysis.

Results

Within two hours of exposure to treatment conditions, cells were noted to lose adherence to plastic when treated with osteoarthritic synovial fluid, while control wells maintained adherence. (Figure 2.1) Cells exposed to medium or normal synovial fluid had no significant difference in viability. Cells treated with any condition of osteoarthritic synovial fluid had significantly different viability than medium or normal synovial fluid. A significant difference was found amongst many of the dilutions of osteoarthritic synovial fluid after a 2-3-fold dilution. (Figure 2.2) Linear regression analysis indicated that at a sixteen-fold dilution the viability of cells would be equivalent to the control population ($r^2=0.81607$, $y=0.0465x + 0.1767$).

Discussion

Our null hypothesis was that exposure to synovial fluid from an osteoarthritic joint would not reduce canine ASC viability. Our results indicate that this is a plausible theory. The cytotoxic contents of OA synovial fluid and their destructive effects on the joint environment make the findings of this paper intuitive. Placing cells into this toxic environment seems counterproductive to our goals in therapy, thus diminishing this cytotoxic environment prior to administering cells may be preferable.

While loss of adherence to the cell culture dish is not an objective measure of cell viability, it does reflect disruption in culture homeostasis. This change was noted within a few hours after exposure to synovial fluid, which may be an indication that response to exposure to synovial fluid is rapid. It would be of interest in a future study to assess the longevity of cell viability following exposure to synovial fluid, by evaluating viability at staggered time points. Limited synovial fluid sample availability prevented this assessment in this study.

After twelve hours of exposure to normal synovial fluid, there was no significant difference in cell viability compared to cells that remained within culture medium. This assessment occurred at a single time point after exposure, which does not give us the ability to predict the longevity of cells within normal synovial fluid. Longer exposure may result in lower viability, particularly in an in vitro environment, where synoviocytes or local stroma is not present to provide nutrients and metabolites necessary for normal cell physiology. Within the study time frame, a significant difference was noted between any sample treated with osteoarthritic synovial fluid and normal synovial fluid or medium

treated cells. This suggests the contents of osteoarthritic synovial fluid contain components that contribute to cytotoxicity.

One possible explanation for reduced cell viability in osteoarthritic synovial fluid would be cell-to-cell interactions between ASCs and cells contained within the osteoarthritic synovial fluid. While this could occur with an intra-articular administration of an ASC treatment, the authors were interested in the cytotoxic effects of synovial fluid without cell-to-cell interactions. In order to eliminate this possible scenario, all synovial fluid samples were centrifuged with supernatant removed from any pellet produced. To further ensure no viable cells could mount a cytotoxic effect on ASCs through a direct cell-to-cell interaction, all synovial fluid samples went through multiple freeze thaw cycles. [123] The presence of cells would be expected within a normal osteoarthritic joint environment, but their interaction with ASCs has not been characterized well in vitro. There is much evidence that ASCs have the capacity to immunomodulate their environment, so these cells may not create much of a threat to the ASC viability. [16-18,96,124] We predicted the cytokines and protein contents present in osteoarthritic synovial fluid would be cytotoxic to the ASCs and wanted to eliminate the possibility of host cells confounding results. There are multiple possible factors that may contribute to cytotoxicity of the ASCs. [113,115,116,125-127] Determining which factors, or combination of factors was beyond the scope of this study.

The length of time ASCs need to be present and viable at the site of injury has not been established, and is likely highly variable dependent on disease and therapeutic effect. Given the capacity for stem cells to provide trophic effects on their environment and local

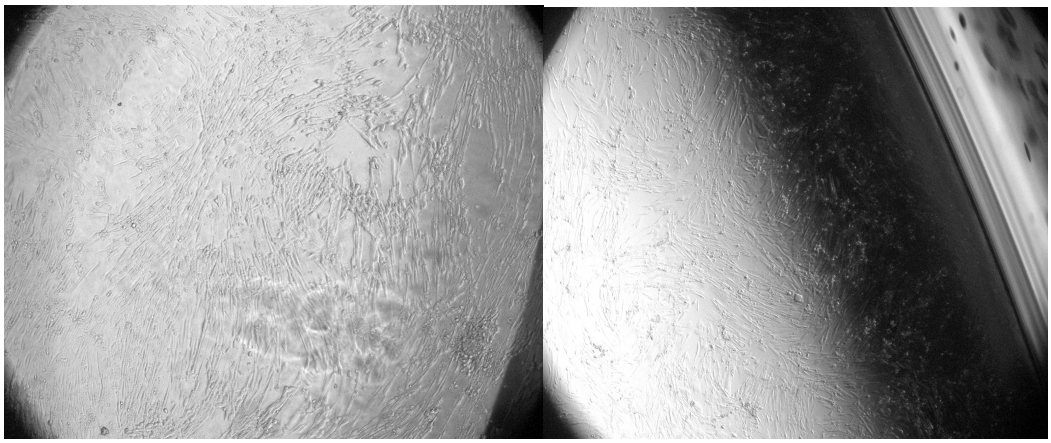
cells, it is plausible that it is not necessary for them to survive more than a few hours to have a positive therapeutic effect. [97,128]

A statistically significant difference between the 1:10 dilution and the undiluted sample, as well as both controls were found. (Figure 2.2) This indicates that while dilution does improve viability, it would take more than a ten-fold dilution to return to an equivalent viability as a healthy joint environment. The authors were curious at what dilutional factor this would be equivalent, to determine if it was practical for possible alteration of administration techniques. Using linear regression analysis, it appears we can predict with confidence that a sixteen-fold dilution would be required. To accomplish this in an in vivo environment, a reasonable approach would be to flush the affected joint with saline prior to administering the ASC treatment intra-articularly. A study comparing osteoarthritic joints that receive ASCs with flushing prior to administration and without flushing would be prudent to determine the necessity and influence on outcome.

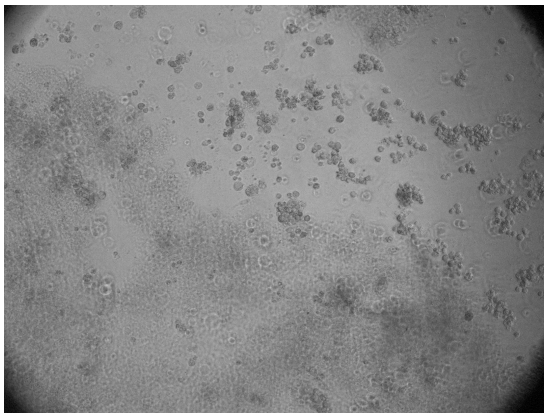
This study is an in vitro study, which limits our ability to translate findings to an in vivo environment. While we have established that osteoarthritic synovial fluid is cytotoxic, we cannot recreate the stromal environment of a joint, which could play a role in providing cytoprotective support to transplanted cells. The best method of evaluating the effect this could have on cells would be to do an in vivo cell tracking study that allows assessment of cell viability. This remains extremely challenging at this stage, subject to many pitfalls. An indirect method of assessing this effect could be to evaluate patient response to ASC administration intra-articularly versus intravenously versus peri-articularly. This

may also address the question of whether it is of clinical significance that viability is reduced and short-lived.

The results of this study indicate that osteoarthritic joint fluid has a cytotoxic effect on ASCs. This suggests we should re-evaluate if the current method of administration is appropriate and if revision of current protocols could improve current therapeutic response. Further investigations in vivo assessing the response to joint fluid dilution or intravenous administration should be undertaken.



Medium **Normal**



OA

Figure 2.1: Images of cell culture wells two hours after exposure to control (medium or normal synovial fluid) or osteoarthritic (OA) synovial fluid. Note the rounded up detached appearance of cells in the OA treated group, as apposed to the spindle shaped appearance of cells of medium or normal synovial fluid treated wells. Microscopic magnification of 10x.

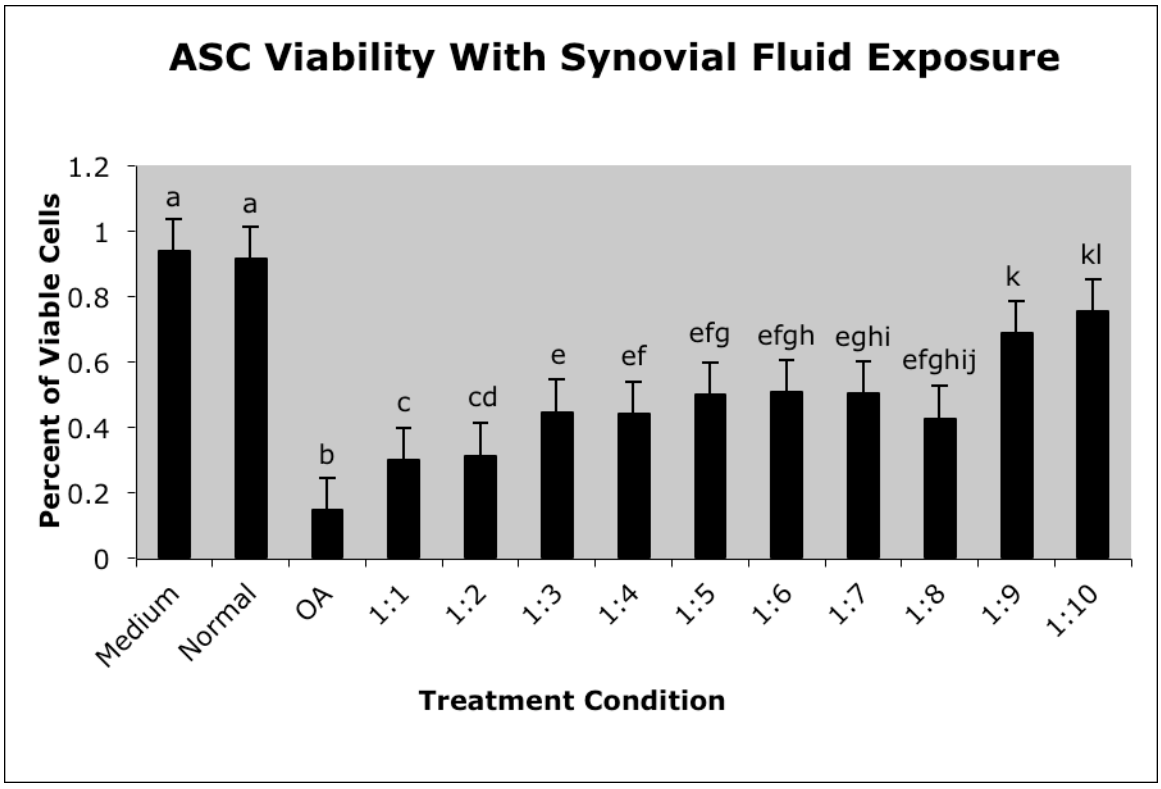


Figure 2.2: Viability of cells after exposure to osteoarthritic synovial fluid as an undiluted sample (OA), or an osteoarthritic synovial fluid sample diluted from 1:1-1:10, or normal synovial fluid or medium alone. Values are expressed as the number of viable cells divided by the total number of cells in the sample, or percent of viable cells. Statistical significance is set as $p<0.05$. Conditions labeled with the same letter have no statistical difference, whereas those with different letters are statistically different.

Chapter 3

The influence of culture medium type on cellular phenotype of canine adipose derived stromal cells.

Introduction

Mesenchymal stem cells have been identified and isolated from multiple adult tissues. [3,15] They provide an ethically acceptable source of multipotent stem cells. [1,6] They have been investigated for the therapy of many disease processes in several species. Canine adipose derived stem cells (ASCs) provide a source of mesenchymal stem cells that are potentially beneficial for the therapy of osteoarthritis in veterinary medicine and are in used in clinical practice. [9,10,19,20] Currently, most veterinary therapy utilizes an autologous, stromal vascular fraction (SVF) generated from the patient in need of treatment. [9,10,19,20] Using allogeneic stem cells may provide advantages over autologous SVF, including more efficient, cost effective treatments without the need for a surgical procedure on the patient in need of therapy. Additionally, cultured stem cells can be exponentially expanded, verified that they maintain multipotency, and selected for desirable qualities prior to use. Allogeneic stem cells have been successfully and safely used to treat other disease processes in dogs. [108,129-131]

The use of allogeneic stem cells as a therapy for osteoarthritis (OA) requires the selection of a medium that provides a consistent, desirable cellular phenotype. Supplements to culture medium can greatly influence cellular phenotype, and even differentiate stem cells towards a specific cell type. This raises questions about the role culture medium

may play in altering the behavior of cultured stem cells when compared to SVF cells. [1,3,6,8,18,132]

In OA, many cytokines play a role in inflammation and pain. Thus, if treating OA with ASCs, the cellular phenotype related to expression of these cytokines is intuitively relevant. Anti-inflammatory mediators associated with a potential therapeutic effect for OA, such as tissue inhibitor of metalloproteinase-2 (TIMP-2) and interleukin 1 receptor antagonist (IL-1ra) would theoretically be beneficial, while minimal expression of proinflammatory mediators, cyclooxygenase 2 (COX-2) and interleukin 1 beta (IL-1β), would be preferred. [21,34,75,82,83,133]

Other phenotypic traits that might be important include a reduced capacity to generate a host response by minimal expression of major histocompatibility complex II (MHCII) and retention of major histocompatibility complex I (MCHI). [134] Cell surface markers identified to be associated with mesenchymal stem cells, such as CD44 and CD90, should be present while hematopoietic cell markers, CD34 and CD45, should be absent. [8,18] Additional stem cell markers demonstrated in other stem cell types and species (CD105, CD117, CD133) would be of interest as well. [8,18]Finally, a capacity to generate cartilage would be desirable for OA therapies.

The objective of this work was to assess the variability of cellular phenotypes suspected to play a role in the therapy of OA after exposure to different culture medium conditions. Our null hypothesis was that changes in culture medium conditions would have no influence on cellular phenotype.

Materials & Methods:

Isolation of Adipose Derived Stem Cells

All procedures were performed in accordance with the University of Minnesota Institutional Care and Use Committee (IACUC # 1203A11421). Six, healthy, 1-year old female intact hound dogs were placed under general anesthesia and, using aseptic technique, approximately 50-gms of falciform fat was collected. SVF was generated from each fat sample using 0.075% Collagenase Type I (Gibco, Life Technologies, Grand Island, New York) digestion as previously described. [9,10] Nucleated cells were counted using a hemocytometer and each sample was aliquoted with standardized nucleated cell numbers for phenotypic assessment of SVF and subculture within each cell culture medium condition.

Cell Culture

Five different cell culture medium conditions were used. They were: basic cell growth medium (BGM) consisting of low glucose Dulbecco's Modified Eagle Medium (Gibco, Life Technologies, Grand Island, New York), 10% characterized fetal bovine serum (FBS) (Hyclone, Thermo Fischer Scientific, Minneapolis, MN), and 1% antibiotics (penicillin 10,000 IU/mL, streptomycin 10,000 ug/mL, amphotericin B 25 ug/mL) (Mediatech Inc, Corning, NY); Keratinocyte N acetyl-L-cysteine supplemented (KNAC)

medium consisting of modified MCDB153 medium (Keratinocyte-SFM) (Gibco, Life Technologies, Grand Island, New York), MCDB153 medium (Keratinocyte-SFM) (Gibco, Life Technologies, Grand Island, New York), 2mM N-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO), 0.2mM L-ascorbic acid 2-phophate (Sigma-Aldrich, St. Louis, MO), 0.09mM calcium and human recombinant epidermal growth factor (5ng/mL) (Gibco, Life Technologies, Grand Island, New York), bovine pituitary extract (50ug/mL) (Gibco, Life Technologies, Grand Island, New York), insulin (5ug/mL) (Sigma-Aldrich, St. Louis, MO), hydrocortisone (74ng/mL) (Sigma-Aldrich, St. Louis, MO), 5% FBS (Hyclone, Thermo Fischer Scientific, Minneapolis, MN), and 1% antibiotic (Mediatech Inc, Corning, NY))[18]; Multipotent Adult Progenitor Cell (MAPC) medium consisting of low glucose Dulbecco's Modified Eagle Medium (Gibco, Life Technologies, Grand Island, New York), 40 % MCDB (Sigma-Aldrich, St. Louis, MO), 1% L-Ascorbic Acid (Sigma-Aldrich, St. Louis, MO), platelet derived growth factor (10ng/mL) (R&D Systems, Minneapolis, MN), epidermal growth factor (10 ng/mL) (Sigma-Aldrich, St. Louis, MO), dexamethasone (0.5uM) (Sigma-Aldrich, St. Louis, MO), 1% ITS+ liquid media supplement, (Sigma-Aldrich, St. Louis, MO) 10% FBS, and 1% antibiotics [135]; serum free medium was Stempro MSC SFM Human Mesenchymal Stem Cell Culture Medium (Gibco, Life Technologies, Grand Island, New York) supplemented with 1% antibiotics and xeno-free medium consisting of StemPro MSC SFM Xeno-free medium (Gibco, Life Technologies, Grand Island, New York), supplemented with 1% antibiotics. Stromal vascular fractions from each cell line were placed into each condition at a density of 8,000 nucleated cells/cm^2. Cultures were placed in incubators maintained at 37°C in a

humidified atmosphere with 5% CO_2. After twenty-four hours, the non-adherent cells were removed by aspirating medium and rinsing the cells with 10mL of phospate buffered saline. Fresh culture medium was placed on adherent cells. Culture media were changed every two days, and when 80-90% confluency was reached the cells were detached using Tryple-E (Invitrogen, Life Technologies, Grand Island, New York), rinsed with phospate buffered saline, and viable cells counted using the trypan blue exclusion method. Cells were then plated or frozen in liquid nitrogen for future assessment. The cell lines were expanded for a minimum of three passages when responsive to media conditions. The SVF and each subsequent cell passage in each medium condition were evaluated for multiple phenotypic properties. Population doubling time was calculated at each passage using the following formula: PD= *t*log2/log (number of viable cells/ number of cells plated), where PD is the population doubling, and *t* is the time in culture.

Gene Expression

Proinflammatory (COX-2 and IL-1B) and anti-inflammatory (IL-1ra and TIMP-2) cytokine messenger RNA levels were measured by quantitative real time polymerase chain reaction. Glyceraldehyde-2-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The primers used for amplification, with the exception of TIMP-2 [18], were generated using the Primer 3 v 0.4.0 program and verified for gene specificity using a BLAST program (BLAST, National Center for Biotechnology Information, National Institutes of Health, Betheseda, MD. Available at blast.ncbi.nlm.nih.gov/) and by gene sequencing the product of the PCR reaction (Biomedical Genomics Center, University of

Minnesota Core Factility, Minneapolis, MN). (Table 3.1) Primers were synthesized by a life sciences company (Invitrogen, Life Technologies, Grand Island, New York). Total RNA was extracted using TRIzol Reagent (Invitrogen, Life Technologies, Grand Island, New York). Reverse transcription of 2μg of mRNA was performed with 2μg SuperScript III Reverse Transcriptase, 1 mM dNTPs, 50ng random hexamers, 20mM RT buffer, 5mM $MgCl_2$, 100mM DTT, and 40U of RNaseOUT (Invitrogen, Life Technologies, Grand Island, New York) in a PTC-100 Programmable Thermal Controller (MJ Research, Inc, St. Bruno, Canada) at 65°C for 5 min, 4°C for 15 minutes, 25°C for 10 minutes, 50°C for 50 minutes, and 85°C for 5 minutes. Quantitative RT-PCR was performed with 6uL Syber Green (Invitrogen, Life Technologies, Grand Island, New York), 0.02ug cDNA, and 0.25 μM forward and reverse primers for 40 cycles of 95°C for 15s, and 60°C for 60s in a Mastercycler (MJ Research, Inc, St. Bruno, Canada). Expression values were normalized to the housekeeping gene, GAPDH, and calculated via the $2^{=\Delta Ct}$ equation. [136] Cytokine expression from the SVF was compared to each of the media conditions after passage 3. Cytokine expression was normalized to GAPDH, which was within one CT cycle, and reported as percentage of GAPDH expression.

Immunophenotype

Cell surface marker expression evaluating immunostimulatory potential [MHCI, (H58A) (VMRD inc, Pullman, WA), MHCII (555810) (BD Biosciences, San Jose, California)], mesenchymal stem cell markers [CD44 (BAG40A) (VMRD inc, Pullman, WA), CD90 (DH2A) (VMRD inc, Pullman, WA) and CD105 (Southern Biotech, Birmingham, AL)

(9811-09)], hematopoietic stem cell markers [CD34 (559369) (BD Biosciences, San Jose, California), CD45 (CAD019A) (VMRD inc, Pullman, WA), CD117 (555714) (BD Biosciences, San Jose, California) and CD133 (12-1331-80) (eBiosciences, San Diego, CA)] were labeled with phycoerythrin flourochrome and evaluated by flow cytometry on a BD FACSCalibur instrument (BD Biosciences, San Jose, California) with a 488nm and 633nm laser. Cells were aliquoted to $1x10^5$ cells per cell surface marker and rinsed with FACs buffer (phosphate buffer solution with 2%FBS) than incubated with primary antibody (MHCI, MHCII, CD44, CD45, CD90) for 30 minutes followed by secondary antibody (PE goat anti-mouse Ig, 550589) (BD Biosciences, San Jose, California) for 30 minutes, or a conjugated antibody marker (CD34, CD105, CD117, CD133) for 30 minutes prior to analysis.

Multipotency

Differentiation was initiated following the third passage in each medium condition. Adipogenic differentiation consisted of plating cells at a density of 10,000 cells/cm^2 and exposure of cells to adipogenic differentiation medium consisting of low glucose DMEM (Gibco, Life Technologies, Grand Island, New York) supplemented with 5% FBS, 0.5uM dexamethasone (Sigma-Aldrich, St. Louis, MO), 5uM insulin (Sigma-Aldrich, St. Louis, MO), 10uM indomethacin (Sigma-Aldrich, St. Louis, MO), and 0.25mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO) for three days, then growth medium for three days, cycling for 21 days total. [18,132] Osteogenic differentiation consisted of plating at a density of 1,000 cells/cm^2 and exposure of cells to osteogenic

differentiation medium consisting of low glucose DMEM, 5% FBS, 50nM dexamethasone, and 5mM beta-glycerophosphate (Sigma-Aldrich, St. Louis, MO) with medium changes every three days for 6-8 weeks. [18,132] Chondrogenic differentiation consisted of pelleting 100,000 cells in 10uL of growth medium, and exposure of the pelleted cells to chondrogenic medium consisting of low glucose DMEM, 10% FBS, 10ng/mL TGF-β (*p* Peprotech, Inc, Rocky Hill, NJ), 50uM L-ascorbic acid (*d* Sigma-Aldrich, St. Louis, MO), and 6.25ug/mL insulin (Sigma-Aldrich, St. Louis, MO) with medium changes every three days for 14 days. [132] Each condition was evaluated in duplicate, and compared to a negative control consisting of cells cultured at the same density and conditions with the exception of exposure to growth medium in the stead of differentiation medium.

Evaluation of adipose differentiation was accomplished with oil red O staining (*d* Sigma-Aldrich, St. Louis, MO), alizarin red staining (*d* Sigma-Aldrich, St. Louis, MO) for bone differentiation assessment, and Alcian blue staining and histological assessment was used for chondrogenic differentiation. Histologic assessment was done by one author (TDO) who was blinded to treatment group. Adipose and bone differentiation is reported as a positive or negative result. Cartilage differentiation was scored on histologic assessment according to the following scale: 0= none (no evidence of rounded chondrocyte morphology or production of Alcian blue staining matrix), 1= poor (<10% rounded cells (ie mostly spindle cells) and minimal production of Alcian blue matrix), 2= fair (10-25% of cells rounded (ie many spindle cells) and small amounts of Alcian blue matrix), 3=

good (25-50% of cells rounded and moderate amounts of Alcian blue matrix), 4= excellent (>50% of cells rounded and abundant Alcian blue matrix).

Statistical Analysis

Population doubling times and quantitative RT-PCR data were analyzed using a Wilcoxon signed-rank test, with $p<0.05$ considered statistically significant. All data are expressed as mean ± standard deviation.

Results:

Isolation of Adipose Derived Stem Cells

After harvest and processing, total nucleated cell counts were calculated for each sample. The mean ± standard deviation total nucleated cell count was $5.9 \times 10^6 \pm 4.65 \times 10^6$ cells (range: $2.065\text{-}13.35 \times 10^6$ cells).

Cell Culture

Growth of ASCs in SFM and xeno free conditions was very poor and 80% confluency of cells was not reached in either medium condition. Therefore these conditions did not provide sufficient cell numbers for assessment of any criteria. When comparing population doubling times within a passage, BGM created significantly shorter doubling times when compared to KNAC (p-value=0.028) and MAPC (p-value=0.028) after passage two (mean±standard deviation of population doubling time of all cell lines at passage two for each medium condition: BGM 1.439±0.4457, KNAC 3.316±1.040, MAPC 3.171±2.306). (Figure 3.1)

Gene Expression

Proinflammatory cytokine gene expression significantly decreased in all of the media conditions when compared to the SVF. However, there were no statistical differences in proinflammatory marker expression in any of the media conditions. (Figure 3.2) Anti-inflammatory cytokine expression also significantly decreased in any culture conditions, with no significant differences in expression levels among media conditions. (Figure 3.3) SVF not only expressed significantly higher levels of all cytokines, but variation about the mean was dramatically higher.

Immunophenotype

MHCI expression was present in all conditions. MHCII was expressed in a small population of SVF cells (10.5%) and then significantly decreased once cells were cultured. (Figure 3.4) All medium conditions retained cell populations expressing CD44 and CD90, although BGM conditions retained a larger percentage of CD90-expressing cells. A small percentage of cells (9.5%) expressed CD105 in the SVF, but CD105 expression was lost after culture in any media condition. Similarly, CD34 (44.7%) and CD45 (17.05%) expression was found in the SVF, but not in cell populations after culture. CD133 may be expressed in a very small population of cells in SVF, BMG and MAPC medium conditions. CD117 was not expressed by any population of cells.

Multipotency

All culture conditions that enabled cell expansion also allowed tissue differentiation, but cells in BGM medium did not generate adipocytes or osteocytes. KNAC and MAPC both allowed tri-lineage differentiation. (Table 3.2)

Discussion:

Isolation of canine adipose derived stem cells was successful as previously reported [8,9,18,19,132], but provided variable yields of nucleated cells, indicating variable products. Determining the characteristics to identify individuals that might provide greater yields was beyond the scope of this study. A greater nucleated cell yield does not necessarily provide a greater ASC yield, as the method for counting nucleated cells does not distinguish cell type.

SVF gene expression of pro and anti-inflammatory cytokines consistently decreased after 3 passages in cell culture and achieved similar levels in all culture conditions. This suggests that either the majority of the expression of these cytokines is from cells that are lost from the SVF in cell culture and are not present in the ASC or that each of the culture conditions resulted in similar gene expression in cells comprising the ASC. Isolation of the specific cell type(s) from the SVF that result in the preferential expression of either pro or anti-inflammatory cytokines would be useful.

Stromal vascular fractions contain a heterogeneous population of cells, including red blood cells, leukocytes, adipocytes, and a small population of ASCs. [137] This may be an explanation for the variations in expression of surface markers and cytokine expression in

SVF populations. Of these cell populations, only the progenitor cells and stromal cells should be capable of self-renewal, eliminating much of the heterogeneity of cell type by culturing. This is a possible explanation for the apparent loss of cell populations in the FACS data following cell culture, specifically the CD34+, CD44-, CD45+, CD105+, and MHCII+ cells that were seen in the SVF. Alternatively, the change in expression may be due to a change in phenotype of a persistent population of cells. Similar loss of cytokine expression in passaged cells may be explained by the same mechanisms. Culturing the SVF appears to generate cells of a more uniform phenotype, regardless of the donor or medium type.

Identifying individual cell populations as ASCs is difficult, as surface markers commonly used to identify ASCs are not specific for that population. CD44, CD90 and CD105 are considered to be markers of mesenchymal stem/stromal cells but may be found in many differentiated mesenchymal cells across many species. However, CD105 was not expressed on canine ASCs in a previous study; a finding that was corroborated in our study. [8] It is interesting to note that a small percentage of cells appeared to express this marker prior to culture. The presence of some cells expressing hematopoetic cell markers (CD45, CD34) is not surprising in the SVF due to the heterogeneous nature of this cell population.

The variable nature of SVF makes the concept of culturing cells to generate a consistent phenotype and cell population appealing. The cultured cells could be used either as an autologous or allogeneic product. The ability to expand the cells in culture would also facilitate the treatment of multiple individuals with a single, characterized product. In our

study, SFM and xeno-free medium conditions were unsuccessful for expanding canine cell populations, in spite of success in other species. A recent study identified an alternate serum free medium supplemented with a serum substitute that was successful in providing adequate proliferation in canines. [138] The elimination of fetal bovine serum from the culture medium would be ideal, due to the highly variable content of this material, and thus the potential for inconsistent phenotypes of cells, and concerns that the xenobiotic material that may induce an immune reaction. Therefore, even though the SFM tested in this study was not found suitable for expansion of canine ASCs, the pursuit of SFM for this purpose should not be abandoned, and appears possible with the correct conditions. Canine serum could be considered an alternative and evaluated for the purposes of growth expansion, but may still be variable in product content.

The immunogenic potential of allogeneic ASCs in transplants has been raised, but in studies thus far, appears to be feasible for clinical use. [18,108,124,139] An immune response relies, in part, upon the expression of MHCII on the cell surface, but the absence of MHCI on cell surfaces will also elicit an immune response. [134] Our study demonstrated that the level of MHCII is diminished following culture in all populations of cells, while expression of MHCI in low levels are retained. Thus, culturing may not increase an immune response.

It is interesting to note that cells cultured in BGM had efficient population doublings (Figure 3.1) and retained ASC markers CD44 and CD90, but had a poor capacity to differentiate (Figure 3.5). In contrast, KNAC and MAPC cultured cells had longer doubling times but retained the capacity to differentiate into multiple lineages.

Differences in the expansion medium composition may explain these variations by promoting alternate phenotypes among the cells or by selecting for cells with differing characteristics. Notably, the differences between phenotypes of cells cultured in KNAC and MAPC were minimal.

The low number of cell lines investigated is a limitation of this study. In addition, it is important to note that variation in the source and supply of reagents, particularly fetal bovine serum, may alter phenotype. The FBS used in this study was from a single lot to minimize variability. A further study investigating variability of phenotypes when exposed to different lots and sources of FBS would be prudent. Alternatively, a standard set of phenotypic characteristics selected as ideal for the therapeutic application could be utilized to establish an acceptable phenotype against which all new lots of FBS be measured.

Culture of canine adipose derived stem cells led to a different phenotypic profile than SVF. The type of culture medium had an effect on phenotype, so we reject our null hypothesis that change in culture medium conditions would have no influence on cellular phenotype. However, with the exception of the capacity for the cells to differentiate, the differences were minimal. The selection of culture medium should be made based upon the desired therapeutic application and the qualities pertinent for that particular use. Allogeneic stem cell therapy appears feasible and warrants in vivo investigation.

Primer Sequences			
Name	*Sequence (5'->3', forward/reverse)*	*Expected size (bp)*	*Reference*
COX-2	TGAGCACAGGATTTGACCAG/CAATGTTCCAGACTCCCTTGA	190	This study
IL-1B	CTGATGGCCCTGGAAATGT/GGCTTCTTCAGCTTCTCCAA	160	This study
IL-1ra	TGCAGGTGTCCTCTCAGCTA/GAGCCTGGTCTCATCTCCAG	214	This study
TIMP-2	ACGCGGACGTAGTGATTAGG/TTCCCGCAATGAGATACTCC	184	Kang, et al
GAPDH	GCCAAGAGGGTCATCATCTC/CTTTGGCTAGAGGTGCCAAG	220	This study

Table 3.1: Primers sequences used in this study.

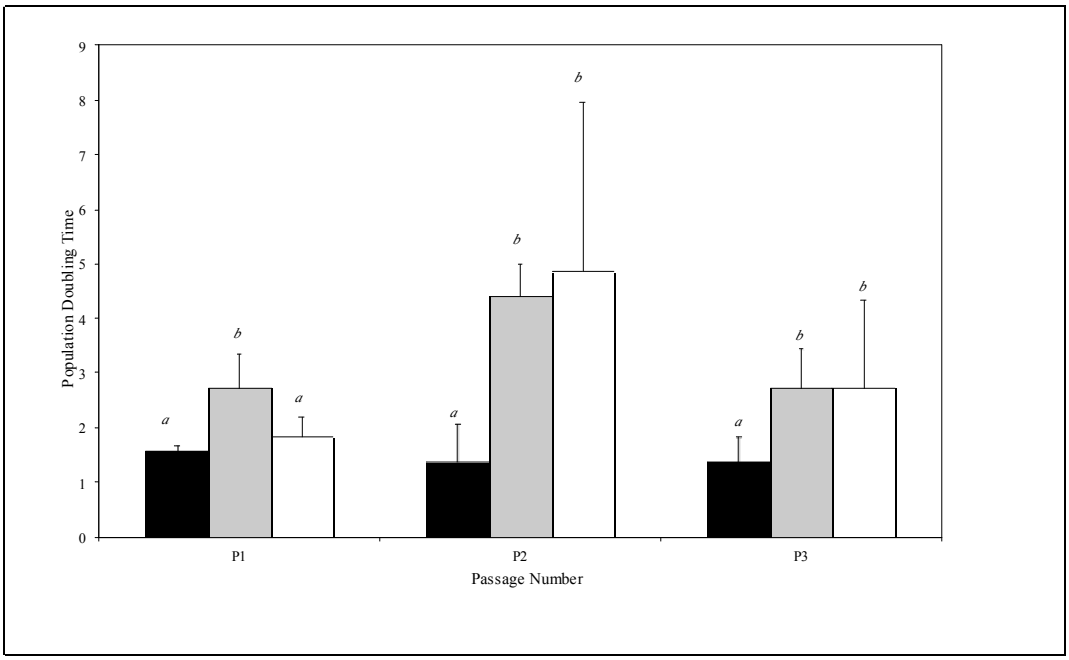


Figure 3.1: Average population doubling time of all cell lines (n=6) in each culture medium condition at each passage (P). Time is expressed as days. Medium conditions consisted of basic growth medium (BGM; black), Keratinocyte N-acetylcholine medium (KNAC; grey), multipotent adult progenitor cell medium (MAPC; white). Conditions with a different letter indicate significant differences in population doubling times within a passage; not between passages.

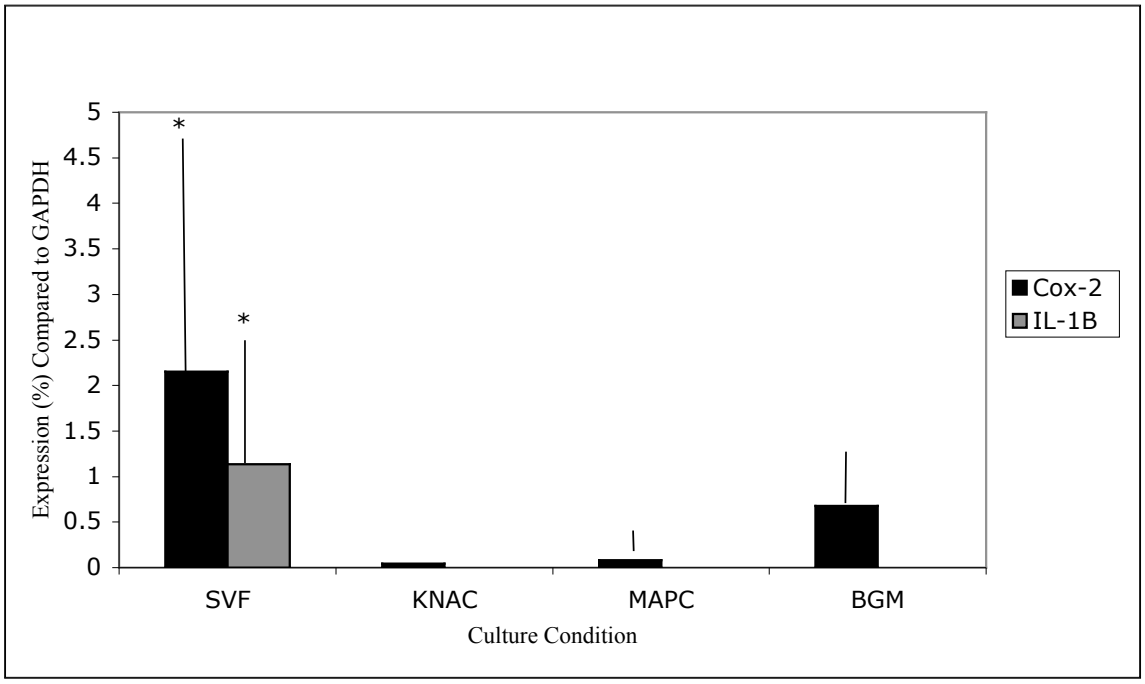


Figure 3.2: Gene expression of proinflammatory cytokines, COX-2 and IL-1B, at initial harvest (SVF), and at passage 3 following exposure to each culture condition consisting of basic growth medium (BGM), keratinocyte N-acetylcholine medium (KNAC), multipotent adult progenitor cell medium (MAPC). Cytokine gene expression was measured with quantitative RT-PCR, and expression values were normalized to the housekeeping gene, GAPDH, and given as a percentage of expression. An asterisk (*) indicates a significant difference between SVF and passage 3; there were no differences among media conditions.

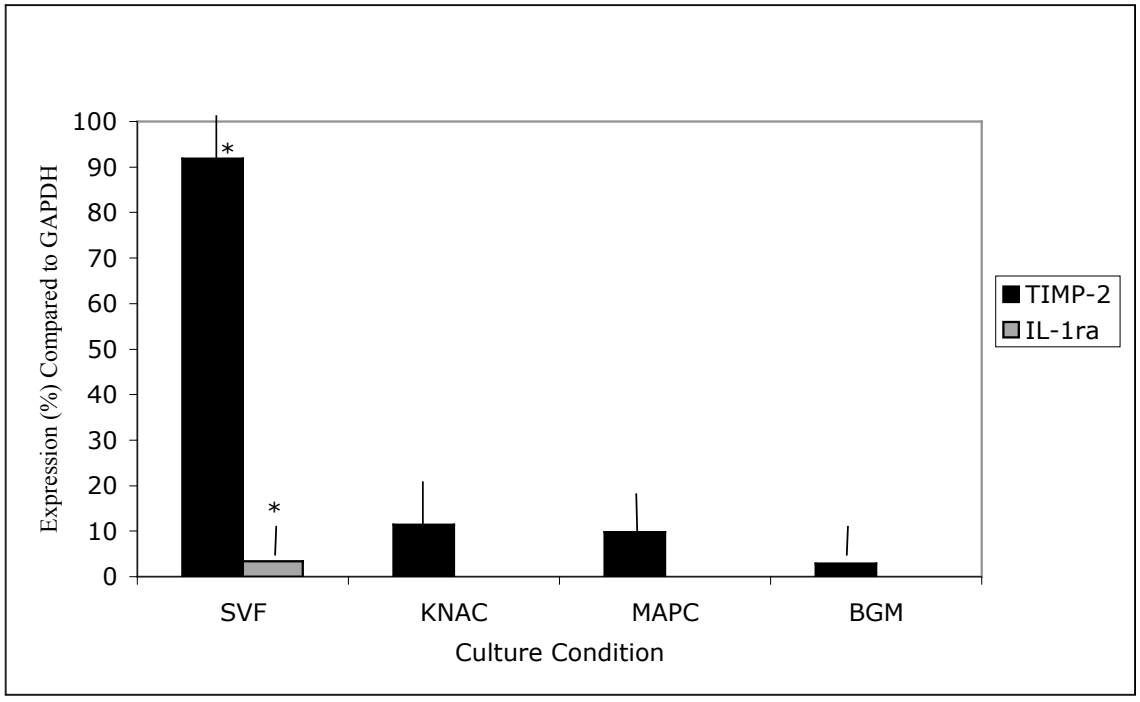
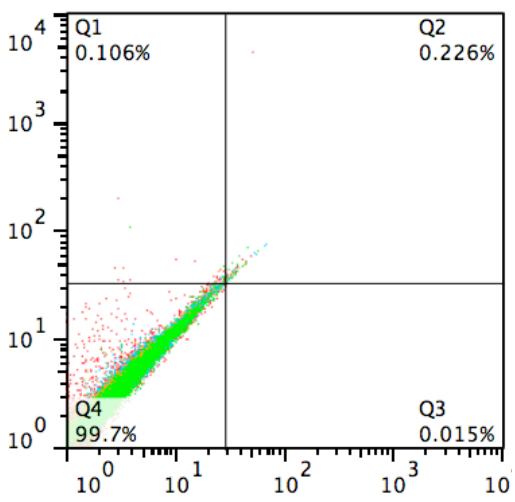
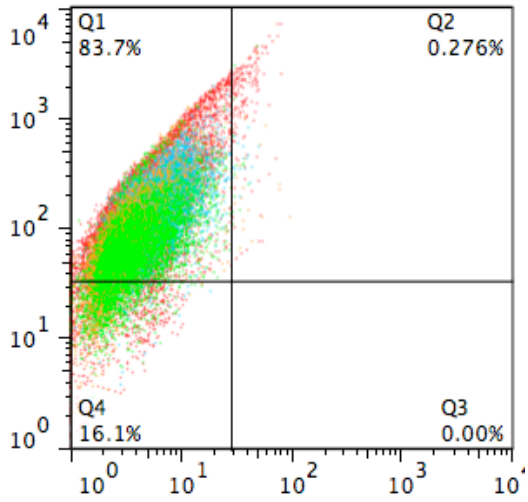


Figure 3.3: Gene expression for anti-inflammatory cytokines, TIMP-2 and IL-1ra, at initial harvest (SVF), and at passage 3 following exposure to each culture condition. Cytokine gene expression was measured with quantitative RT- PCR and expression values were normalized to the housekeeping gene, GAPDH, and reported as a percentage of expression. An asterisk (*) indicates a significant difference between SVF and passage 3; there were no differences among media conditions.



Isotype Control

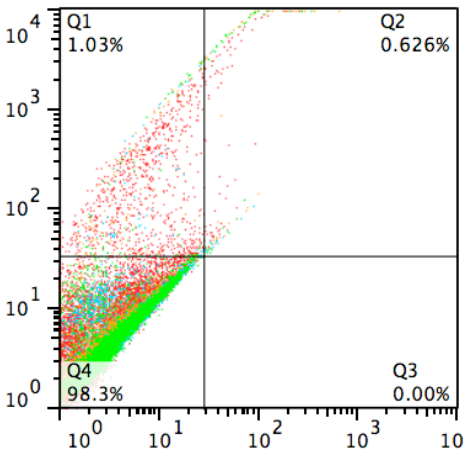


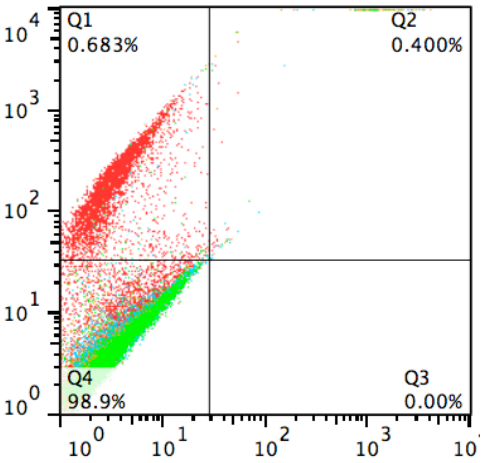
MHCI

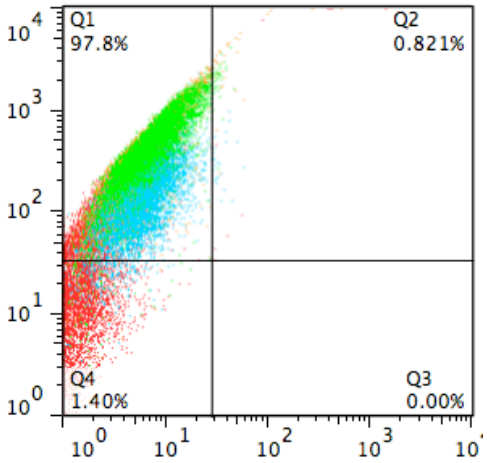
Condition	
	SVF
	BGM
	KNAC
	MAPC

MHCII

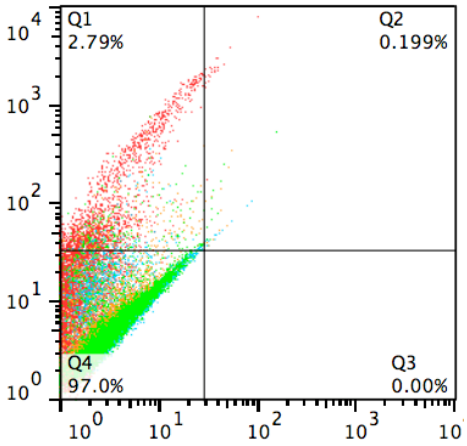
CD34







CD44

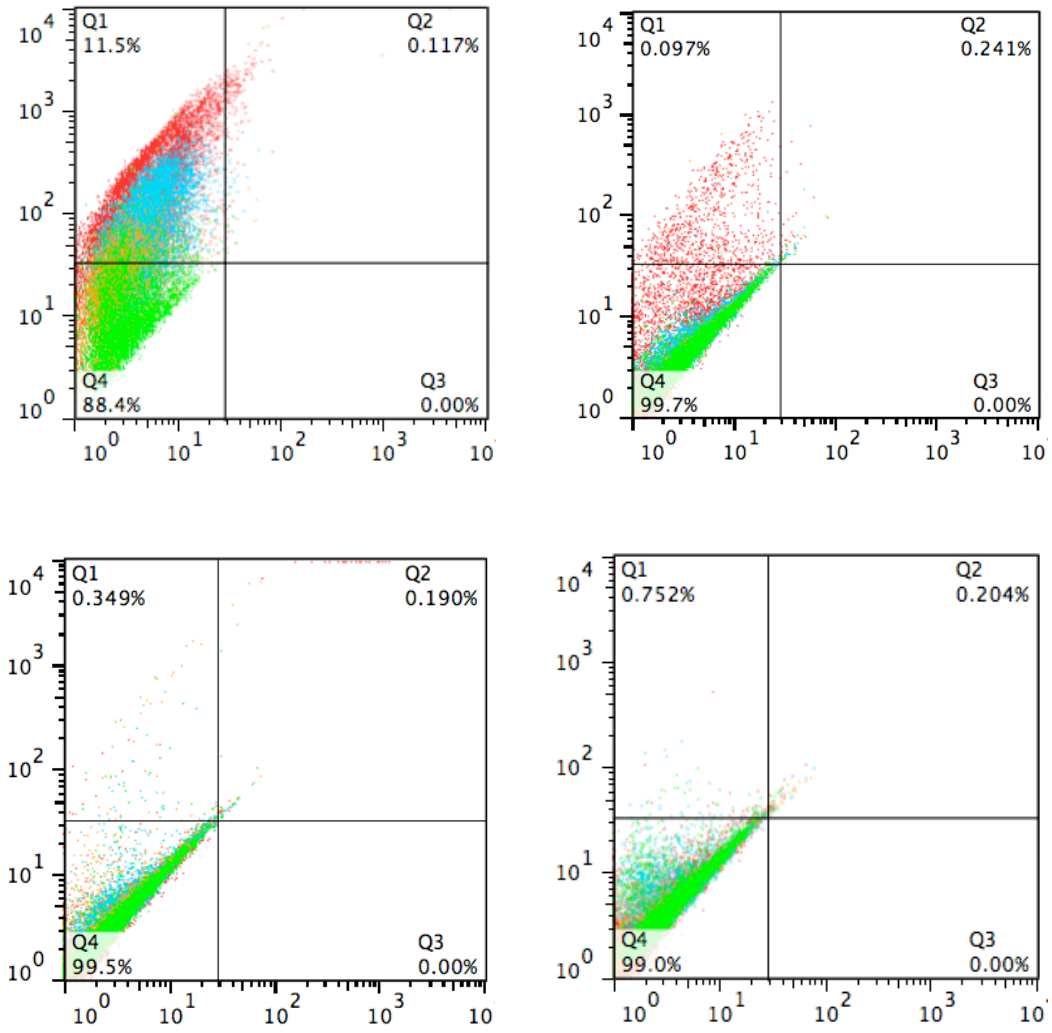


CD45

Condition	
	SVF
	BGM
	KNAC
	MAPC

CD90

CD105



Condition
SVF
BGM
KNAC
MAPC

Figure 3.4: Cell surface markers as analyzed by flow cytometry, depicted as dot plots, which each culture condition plotted within each graph. Each individual graph represents the cell surface marker it is labeled with. Each graph is a representative expression of all samples, depicting a single cell line as a stromal vascular fraction (SVF, red), and in each

culture medium condition, basic growth medium (BGM, blue), keratinocyte N-acetylcholine medium (KNAC, orange), and multipotent adult progenitor cell medium (MAPC, green). The graphs depict the level of fluorescence detected by the FL1 detector on the x-axis, and the FL2 detector on the y-axis. All cell surface markers were labeled with phycoerythrin (PE), so would be found within the upper left quadrant if present on the cells. Cells found in the lower left quadrant would be considered negative for expression. Numerical values listed within each quadrant is the percent of cells present within that quadrant.

Condition	Line	Adipo	Osteo	Chondrocytes
BGM	1	-	-	1
	2	-	-	1
	3	-	-	1
	4	-	-	-
	5	-	-	-
	6	-	-	-
MAPC	1	+	+	-
	2	+	+	3
	3	+	contaminated	-
	4	+	+	-
	5	+	+	3
	6	+	+	-
KNAC	1	+	+	-
	2	+	+	3
	3	+	contaminated	2
	4	+	+	3
	5	+	+	-
	6	+	+	-

Table 3.2: Differentiation potential for each cell line in each medium condition consisting of basic growth medium (BGM), Keratinocyte N-acetylcholine medium (KNAC), multipotent adult progenitor cell medium (MAPC). Cartilage differentiation

(chondrocytes) is graded as 0=none, 1=poor, 2=fair, 3=good, 4=excellent, and adipocyte (adipo) and osteocyte (osteo) conditions are listed as positive or negative differentiation. A negative score for cartilage differentiation indicates no generation of cell pellet for histologic analysis.

Chapter 4

Autologous and Allogeneic Stromal Cells as Adjuvant Therapy for Osteoarthritis Caused by Spontaneous Fragmented Coronoid Process in Dogs

Introduction

Canine osteoarthritis has afflicted many pets and veterinarians who struggle to treat the non-reversible, progressive and painful disease. The disease process can significantly impact quality of life, and concurrent disease process may limit the pharmaceutical options that can be chosen to manage the disease. Because of this, alternative therapies have generated a lot of interest and widespread use, often without adequate evidence based medical information.

Adipose derived stromal cells (ASC) have garnered a lot of interest for the treatment of osteoarthritis, and following a small number of publications, has become a widespread therapy offered to owners. [9,10,19] While these studies show improvement compared to placebo control groups, they lack objective outcome measures and include small numbers of dogs. To date, lameness has been visually assessed with reported benefits, and improved quality of life. Additionally, therapy has been limited to the use of an autologous stromal vascular fraction (SVF). [9,10,19] In contrast, utilizing a donated, cultured and characterized allogeneic preparation of ASC provides a less heterogenous product that can be defined and selected for specific purposes.

Osteoarthritis is prevalent in almost any joint, and can be secondary to a traumatic event, infection, or orthopedic disease. The behavior and location is highly variable dependent upon the etiology; this variability is a bias that should be addressed in clinical trials. This model has the added attraction of having a poor long-term outcome in spite of current therapeutics, which may provide a more detectable response to therapeutic interventions. [38,39,140,141] Many different therapies have been advocated, but none have resolved the problem. Most commonly, arthroscopic examination and fragment removal has been performed. One of the etiopathologies proposed has been elbow incongruency. [140-145] For this reason, corrective osteotomies have been proposed, but have limited evidence, or investigation. [146]

We used objective assessments of limb function using platform gait analysis to evaluate ground reaction forces (GRF). [147-149] In addition we utilized a magnetic resonance imaging (MRI) generated image to generate a score reflecting the glycosaminoglycan (GAG) content of a joint with delayed gadolinium enhanced magnetic resonance imaging of cartilage (dGEMRIC). [150]

The objective of this study was to assess the therapeutic effect of three treatment modalities for a fragmented coronoid process in the elbows of dogs. We assessed dogs that had a proximal ulnar osteotomy (PUO), autologous ASC therapy, and allogeneic ASC therapy. Our null hypothesis was that the three treatments would not significantly differ from dogs treated with standard of care. We hypothesized that all treatments would be well tolerated with no deleterious side effects.

Material and Methods

Case Selection

Forty client-owned dogs that presented to the University of Minnesota, College of Veterinary Medicine or Fitzpatrick Referrals, Eashing, Gadalming, Surrey, UK, for evaluation of a forelimb lameness with identified elbow pain secondary to a fragmented coronoid process were included in this multi-group, multi-center, controlled, randomized study. Dogs could be of any breed, sexual status or age. Informed owner consent for participation in the study with owner agreement to follow postoperative care guidelines and recheck examinations was required for participation in the study. Dogs that had previous elbow surgery, injectable drug therapy for arthritis (e.g. steroids, hyaluronic acid, adequan) within 30-days of treatment, other systemic illnesses, were less than 10 kgs, or had other, concurrent, related (elbow incongruency, OCD) or unrelated orthopedic or neurologic disease processes were excluded. All study procedures were according to a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC # 1203A11421).

Experimental Groups

The first thirty dogs were randomized into one of three treatment groups: standard of care therapy (SOC), SOC with a proximal ulnar osteotomy (PUO), or SOC+PUO and autologous stromal vascular fraction cell therapy (SVF). The final ten dogs enrolled in the study were assigned to the final, fourth group: SOC+PUO and allogeneic stem cell

therapy (AllSC). Initially, all dogs were included in the randomization procedure but availability to the characterized allogeneic stem cells became delayed so these alternative methods were adopted. All dogs that were to receive cell therapies were treated at the University site in an effort to standardize cell preparation techniques and limit cell viability issues that might be associated with parcel delivery. Standard of care was considered to be arthroscopic examination of the affected joint and fragment removal. Dogs receiving a proximal ulnar osteotomy had an oblique cut made with an oscillating saw in the proximal third of the ulna, angling caudocranial at a forty-five degree angle to the caudal aspect of ulna. Complete transection of the ulna was confirmed at the time of surgery and with radiographic assessment post-operatively. Surgeons participating in the study standardized surgical technique in an effort that dogs would receive similar care regardless of treatment site. Following completion of their elbow surgery, dogs assigned to receive autologous SVF therapy group had a ventral mid-line abdominal incision of 4-8cm in length made between the xiphoid process and the umbilicus. The falciform adipose tissue was identified, exteriorized, and ligated with a circumferential 2-0 PDS suture prior to transection. The falciform tissue, approximately 30-gms, was placed into a sterile, covered container and transferred to the laboratory for processing.

Anesthetic protocols were individually selected and designed by board certified anesthesiologists. Post-operative management included hydromorphone 0.05mg/kg intramuscularly every six hours overnight, and beginning the following morning, oral tramadol 2-4 mg/kg every eight hours for ten days and a non-steroidal anti-inflammatory of the surgeon's choice administered per manufacturer's directions for ten days.

Dogs assigned to either stem cell treatment group were given 0.05mg/kg hydromorphone and either 0.125mg/kg diazepam or 0.002-0.004 mg/kg dexmedetomidine hydrochloride intravenously the morning following surgical intervention. A preparation of either 5x10^6 allogeneic stem cells or 5x10^6 nucleated autologous cells constituted in 0.5mL of sterile saline was injected intra-articularly into the operated elbow. This protocol was repeated once, six weeks post-operatively. SVF cells were cryopreserved after the first treatment, and AllSC were cryopreserved for all injections. All stromal cell preparations had been rinsed three times with sterile saline to remove medium or digestive agents from the preparation prior to administration.

Isolation of Adipose Derived Stromal Cells

Autologous

Falciform adipose tissue was harvested at the time of surgery and was processed according to previously reported protocols for isolation of the stromal vascular fraction overnight. [9,10] The nucleated fraction was used to determine cell number for dosage, and aliquoted into 5x10^6 million cells/ dose. One dose was administered the day after surgery, and all remaining doses were stored in liquid nitrogen until further need. [151,152]

Allogeneic

Falciform adipose tissue was harvested at the time of surgery from ten healthy dogs admitted to the University of Minnesota College of Veterinary Medicine for

abdominal surgery unrelated to the study. Dogs with neoplasia or septic abdomens were excluded. Owner consent was obtained and all procedures were performed in accordance with the University of Minnesota Institutional Care and Use Committee (IACUC # 1203A11421). Adipose tissue was processed according to previously reported protocols for isolation of the stromal vascular fraction. {Black,L.L. 2007; Black,L.L. 2008} The nucleated cell fraction was than placed into Keratinocyte N acetyl-L-cysteine supplemented (KNAC) medium consisting of modified MCDB153 medium (Keratinocyte-SFM) (Gibco, Life Technologies, Grand Island, New York), MCDB153 medium (Keratinocyte-SFM) (Gibco, Life Technologies, Grand Island, New York), 2mM N-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO), 0.2mM L-ascorbic acid 2-phophate (Sigma-Aldrich, St. Louis, MO), 0.09mM calcium and human recombinant epidermal growth factor (5ng/mL) (Gibco, Life Technologies, Grand Island, New York), bovine pituitary extract (50ug/mL) (Gibco, Life Technologies, Grand Island, New York), insulin (5ug/mL) (Sigma-Aldrich, St. Louis, MO), hydrocortisone (74ng/mL) (Sigma-Aldrich, St. Louis, MO), 5% FBS (Hyclone, Thermo Fischer Scientific, Minneapolis, MN), and 1% antibiotic (Mediatech Inc, Corning, NY))[18];This protocol was chosen from amongst the treatments assessed in the previous study, to maximize tri-lineage differentiation, particularly chondrogenesis, and retention of CD90, CD44 and MHCI surface markers, as well as minimize pro-inflammatory mediators. Cells were expanded to provide multiple doses per cell line, and harvest for therapy at passage three. They were than aliquoted as 5 x 10^6 cells per dose and stored in liquid nitrogen until use. [151,152]

Intra-articular injections

All stem cell preparations were rinsed three times with sterile saline to remove medium or digestive agents from the preparation prior to administration. A preparation of either 5x10^6 allogeneic stem cells or 5x10^6 nucleated autologous cells constituted in 0.5mL of sterile saline was injected intra-articularly into the operated elbow 48-hours after surgery. This protocol was repeated once, six weeks post-operatively.

Subjective Outcome Measures

Canine Brief Pain Inventory

Owners completed a Canine Brief Pain Inventory (CBPI) questionnaire prior to surgical intervention, and six months post-operatively. The CBPI is a validated means of assessing owner opinion of outcome regarding pain and activity in dogs with clinical symptoms secondary to osteoarthritis.. [153,154]

Radiographs

Radiographs were taken prior to admission of study to confirm disease diagnosis. Dogs that received a PUO had radiographs taken immediately post-operatively, at six weeks and at six months post-operatively. Each follow-up radiograph was evaluated for healing at the site of PUO. Osteotomy sites were considered healed if the site was bridged with osteosynthesis with little to no evidence of osteotomy site, not healed with osteogenic activity present, or not healed with no osteogenic activity.

Objective Outcome Measures

Limb Function

Force platform gait analysis was performed to measure ground reaction forces. Each dog was assessed prior to surgical intervention, and at six months post-operatively. Data was collected by acquiring five valid trials for each forelimb at a walk on a force platform (AMTI OR 6-5 force platform, Advanced Mechanical Technology, Watertown, Mass.) measuring $0.5m^2$ in the center of a 10m runway. Velocity and acceleration were measured with the aid of five photoelectric cells coupled with a triggered timing mechanism and mounted 1m apart. A velocity range between 1-1.3 m/s, and an acceleration range of -0.5 to 0.5 m/s^2 was necessary to consider a trial valid. Data was collected at 1000 Hz, and stored on a personal computer with software (Sharon Software Inc, Dewitt, Mich.) designed to record values velocity, acceleration, peak vertical force (PVF) and peak vertical impulse (VI). To be considered valid, the entire foot had to make contact with the platform, without striking an edge, and the ipsilateral hind limb needed to follow with the same criteria. All dogs were weighed prior to data collection and trials were normalized for the patient body weight.

Cartilage Quality

All dogs received an MRI with dGEMRIC scores as previously described in the morning, just prior to surgery immediately following. [150] MRI was repeated six months post-operatively. After receiving anesthesia, each patient was administered gadopentate dimeglumine (0.1 mmol/kg) intravenously followed by ten minutes of passive range of

motion of the affected elbow. MRI was performed as previously described, with a 3T system (GE Sigma EXCITE Milwaukee, WI). [150] The initial images were evaluated, and two sagittal slices that included the medial coronoid process were selected for TN-weighted FSE inversion recovery sequences, to allow further processing with a data software program (Matlab, MathWorks, Natick, Mass., USA), to generate a color map for each of the two images (dGEMRIC image). [150] Each dGEMRIC image was than evaluated and a region of interest (ROI) selected. Each ROI was drawn to include articular cartilage of the medial coronoid process, the corresponding articular cartilage of the humerus, and the intra-articular space in between. [150] The ROI was selected by the same individual for all cases and the individual was blinded to treatment group. Each image was evaluated three different times, with a new ROI identified each assessment. The image intensity scores generated by the program from the ROI were averaged for the three images creating a single, average dGEMRIC score for each assessment period.

Statistical Analysis

Statistical analysis was performed using SOFA Statistics software (SOFA, v1.3.4, Paton-Simpson & Associates Ltd, Auckland, New Zealand). CBPI scores are reported as means of pain or function scores. GRF are reported as percentage of body weight (100*N/N) and values are expressed as means of the five collected trials at each assessment. dGEMRIC scores are reported as the mean of the all ROI generated for each outcome measure time point. Summary statistics are all reported as mean +/- standard deviation

(SD). Differences between groups at the initial assessment, and at the 6-month recheck were evaluated using a one-way ANOVA test. Differences between initial exam and 6-month recheck for each treatment was evaluated using a paired t-test. Values of $p \leq 0.05$ were considered statistically significant.

Results

Forty dogs (n=10/group) met the inclusion criteria and completed the study. Body weights ranged from 12.5 kg to 64 kg, (31.82kg+/-9.12), There was no significant difference between any group weights, or between initial assessment of weight and at recheck. Patient age ranged from 0.66 to 10 years (2.47years+/-2.43). The AllSC treated group was significantly older (4.43years +/-3.187, p=0.03) than any of the other treatment groups, while no significant difference in age was found in the other three treatment groups.

Some dogs that received an autologous or allogeneic ASC injection had two to three days of lameness after injection for one to three days that resolved without further intervention. No local swelling or erythematous reactions were noted or reported by owners. No other deleterious effects were noted from ASC administration.

No significant difference was found between group pain and function scores on the CPBI scores at the initial assessment. At the six month recheck, the AllSC treated group had a statistically significantly higher pain score (5.875+/-4.23, p=0.048,0.028) than the standard of care and SVF group, but not the PUO group. There was not a significant difference amongst the other group pain scores, or any of the function scores. (Figures

4.1 and 4.2) There was a significant decrease between initial pain score and 6 month recheck pain score, and initial and 6 month function scores in all groups. (p<0.001). All dogs with a PUO had some osteosynthesis present at the 6-week recheck. 28/30 dogs were considered healed at that assessment. Of the two remaining dogs, one was considered healed at the 6-month recheck, and the final one was considered healed at a one-year recheck. Subjectively, all dogs had some shifting of the proximal ulna relative to the distal ulna on recheck radiographs, as compared to the post-operative radiographs. There no significant differences in the GRF between any of the groups at the initial assessment (PVF p>0.065, VI p>0.093 for all groups) and at the 6-month assessment (PVF p>0.26, VI p>0.376). (Figures 4.3 and 4.4) All groups had an improvement in limb function and GRF between initial and 6-month recheck. There was not statistical significance in VI in any of the groups (p>0.068), but a statistically significant improvement was found in PVF of the control group and the SVF group (p=0.006 and p=0.002, respectively).

There was no significant difference in dGEMRIC scores amongst groups at initial assessment (control 354.65+/-45.52, PUO 306.93+/-62.141, SVF 337.43+/-39.19, AllSC 358.7+/-27.56), or at the 6-month assessment (381.6+/-65.49, 359.15+/-53.88, 329.37+/-50.49, 365.5+/-60.83, respectively). A significant difference was identified between the initial score and the 6-month score in the control group and the PUO group, with the average 6-month score being higher. (Figure 4.5)

Discussion

To date, there are no published studies evaluating the use of AllSC to treat osteoarthritis in dogs, in spite of the many advantages they provide over SVF. We undertook this study to evaluate safety and efficacy utilizing objective outcome measures. To provide consistency in our assessments (and since the number of dogs/group were small), we elected to investigate a single disease process, fragmented coronoid process (FCP), which inevitably results in osteoarthritis of the canine elbow. [140]

The mean age of the dogs enrolled in the study is anticipated, given the disease process. [140,142,155] The allogeneic group overall had dogs with an older age, but in particular included the two oldest dogs enrolled, at 8 and 10 years at time of enrollment. These two cases likely skewed the mean age of this group relative the other three groups. This may have skewed the outcome of this group, as the longevity of the disease process may increase the severity of osteoarthritis and thus limit the ability to improve,, [140,142,155] although identifying an age correlation to severity of osteoarthritis was not within the scope of this study to assess. Severity of osteoarthritis may be a factor in response to therapy. A future study assessing response across age groups, or response according to cartilage quality would be a good follow-up to assess this problem.

A minor complication associated with ASC delivery was noted in some dogs, and was self-resolving and self-limiting. Owners reported after the second treatment that dogs became more lame than prior to injection, but that it resolved within two to three days. It is difficult to determine within this study if this is secondary to the ASCs, or secondary to an intra-articular injection. A study comparing ASC injection to saline injection would

be necessary to answer this question. There was no reaction to injections that would correlate with a severe immune response, which suggests that ASC therapy, as an autologous or allogeneic preparation, is likely safe to use intra-articularly. It is possible that a systemically administered preparation (intravenous) could result in a different outcome.

All dogs had improved CBPI scores from initial assessment to six-month rechecks, which could be explained by true improvement, or perceived improvement. This outcome measure was the most subjective of our assessments. Unfortunately, owners were not blinded to the group they were assigned to, as it was apparent which groups had an abdominal incision. We did not feel it was ethically appropriate to perform surgical incisions in all dogs only in order to blind the owners. Likewise, dogs receiving a stem cell injection were sedated at the second injection, whereas the other groups were not, making it apparent to owners which group they had been assigned to. Control group dogs did not require radiographs, and this was apparent on the itemized bill that owners would have received a copy of, thus making it possible for the astute owner to deduce if they had been assigned to the PUO group. This is important to note, as the care-giver placebo effect can be quite potent. [156] The AllSC group had improvement of function based on scores, but pain scores at the 6-month recheck were higher compared to the control group and SVF group. It should be noted that two owners within this group neglected to turn in the 6 month CBPI questionnaire, and would not respond to requests for completion. A third owner in this group scored the function portion of the questionnaire at the initial assessment, but chose to write comments in place of scoring the pain portion, and was not

available for follow-up clarification. This could have skewed the data significantly, as the numbers were much lower than the other groups, which had 100% compliance. It should be noted that they were still significantly lower than at initial assessment. Radiographs were assessed in each group receiving a PUO six weeks after surgery to ensure healing and shifting. The only noted problems with the PUO were delayed healing in the two older dogs. Radiographic union did not appear to correlate with function, as both of the older dogs were within the allogeneic group, which did not have significantly different GRFs from the other groups.

Ground reaction forces improved after intervention regardless of group, but were only statistically significantly different in the control and SVF groups. The ground reaction forces of these groups were lower at initial assessment, which may have allowed for a greater degree of improvement, and thus statistical significance. It cannot be ruled out that initial GRFs are predictive of outcome regardless of therapy.

Cartilage quality is a difficult thing to assess objectively and non-invasively, and dGEMRIC holds a lot of promise for resolving this problem. One would anticipate that a positive response to therapy would equivolate to a static dGEMRIC score, rather than a decreased score, which would be a reflection of continued cartilage degeneration. One would expect with the known pathology of FCPs that the dGEMRIC score would decrease over time, as the disease is progressive. None of our treated groups had a decrease in score, however the control group and PUO group had an increase in score. Since OA progresses radiographically after arthroscopic debridement of a FCP we would not predict this. Explanations for this finding include that the follow-up time (6-months)

was too short and arthritis would have progressed if follow-up time were longer, or that proteoglycan content in the medial compartment in fact does not decrease following surgery yet osteophytosis as seen with radiographs does. The dGEMRIC procedure had good inter- and intra-observational consistency when evaluated in normal dog elbows. [150] While it has not been applied to abnormal dog elbows, in a clinical situation, prior to this study it has been used in to follow the progression of OA in people. [157,158] In addition, dogs with dysplastic elbows often have very distorted anatomy, either from blunted coronoid processes, osteophyte accumulation, or fragmented and possibly even displaced coronoids. Following coronoidectomy, the landmarks are even more distorted, making it possible that slice selection poses a much greater challenge in post-operative patients. If slice selection is inconsistent from pre-operative to post-operative assessment, one could get a significantly different cartilage score from one time to another. Likewise, even if slice selection is consistent, coronoidectomy changes the anatomy that is evaluated and, in some cases, may change the proteoglycan content in the ROI. For example, in dogs with a FCP, the fragment has the greatest amount of diseased cartilage and in some cases the region of the ulna with the fragment may have no cartilage at all. After this diseased region is removed the remaining components of the medial compartment would have a higher average proteoglycan content. If this is true one could argue that dGEMRIC shortly after surgery should have been compared to the 6-month follow-up; that comparison would likely provide for a more accurate assessment of the response of the cartilage to the intervention over time.

Standard of care therapy (arthroscopy and fragment removal), has recently been questioned as an effective means of therapy, as conflicting reports have been published.[38,39,142,144] We may have found a greater improvement with groups if compared to a group that received no surgical intervention. Unfortunately, we were limited by case availability, so had to limit the number of groups we could assess. This particular disease process is complicated, and not fully understood, which may have biased our groups, making detection of improvements difficult.[140,142,143,145] Those that are admitted to the study with higher levels of performance and lower levels of pain may do better long-term regardless of therapy. Also, patients that are admitted with severe lameness and pain have greater room for improvement. We did not detect a significant difference between PUO and the control group, as has been previously reported.[146,159] We did not select cases for the PUO based on degree of incongruency, nor did we select for younger dogs, as these dogs were randomized into their group. It may be that a PUO would be effective and beneficial under specific criteria that we did not select for.[146,159] Future studies should consider stratifying groups according to initial outcome measures to alleviate some of the bias that appeared within our groups.

We achieved long-term outcome measures, with assessments and follow-up through 6 months following intervention, but it may be argued that the therapeutic benefit of ASCs could be much farther reaching than six months, and the greatest difference may be noted if we had the capacity to follow cases out several years.

We identified no contra-indications to utilizing autologous or allogeneic ASC therapy, and have provided some evidence that there may be some benefit. Allogeneic had similar

functional scores to other treatments, but will require further investigation to determine if this is secondary to bias or an accurate reflection of therapeutic effectiveness. We can also conclude that dGEMRIC has promise for future applications as an objective assessment of cartilage quality, but will require more stringent guidelines and attention to slice selection and ROI.

One major limitation of this study is the small numbers of dogs in each group. A greater number may provide more clarification in differences amongst groups, and possibly eliminate some of the bias in initial group function. Given the multiple groups, we could not expand the number of dogs per group within this study.

We cannot reject our null hypothesis for PUO or autologous ASC therapy, when compared to the control group. We could reject our null hypothesis for allogeneic ASC therapy based upon GRF significance, but suggest further assessment based on biases. We accept our hypothesis that all therapies are safe and well tolerated.

This study provides preliminary data for the safety of autologous and allogeneic ASC therapy, and should lead to further studies with greater numbers of dogs, with stratification of participants based on initial function and dGEMRIC scores.

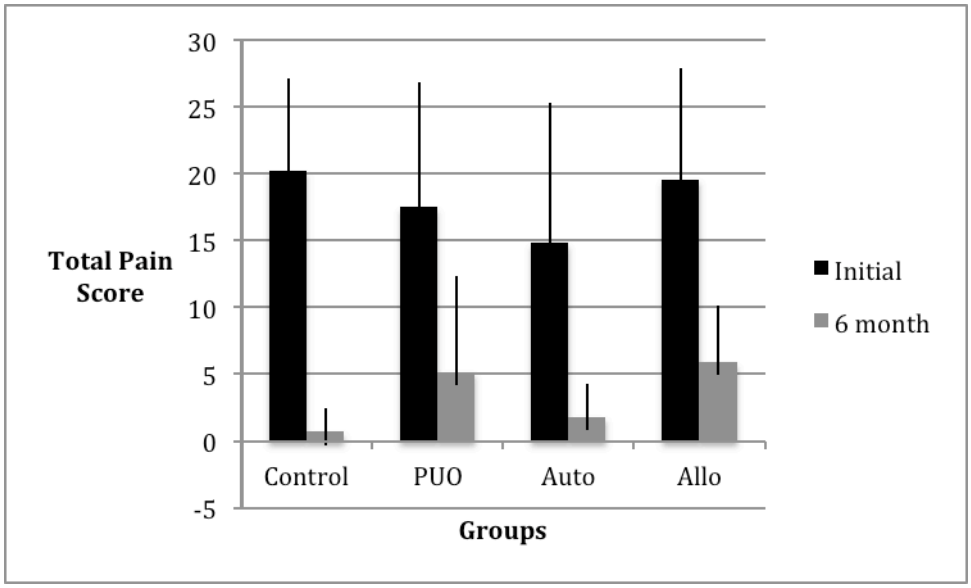


Figure 4.1: Average score of canine brief pain inventory questionnaire for each treatment group at initial assessment (black), and at 6-month follow-up assessment (grey). The x-axis is each treatment group, and the y-axis is the averaged score of owner assessment of patient pain calculated by answering four questions. (0= no pain, 10= extreme pain) The highest (worst pain) possible score would be forty.

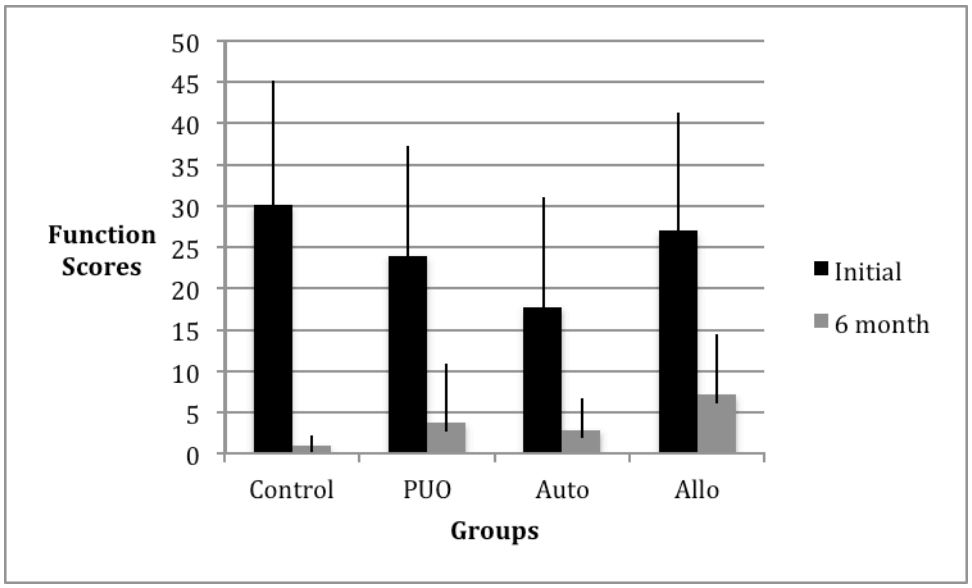


Figure 4.2: Average score of canine brief pain inventory questionnaire for each treatment group at initial assessment (black), and at 6-month follow-up assessment (grey). The x-axis is each treatment group, and the y-axis is the averaged score of owner assessment of patient function calculated by answering six questions. (0= no interference, 10= completely interferes) The highest (worst) possible score would be sixty.

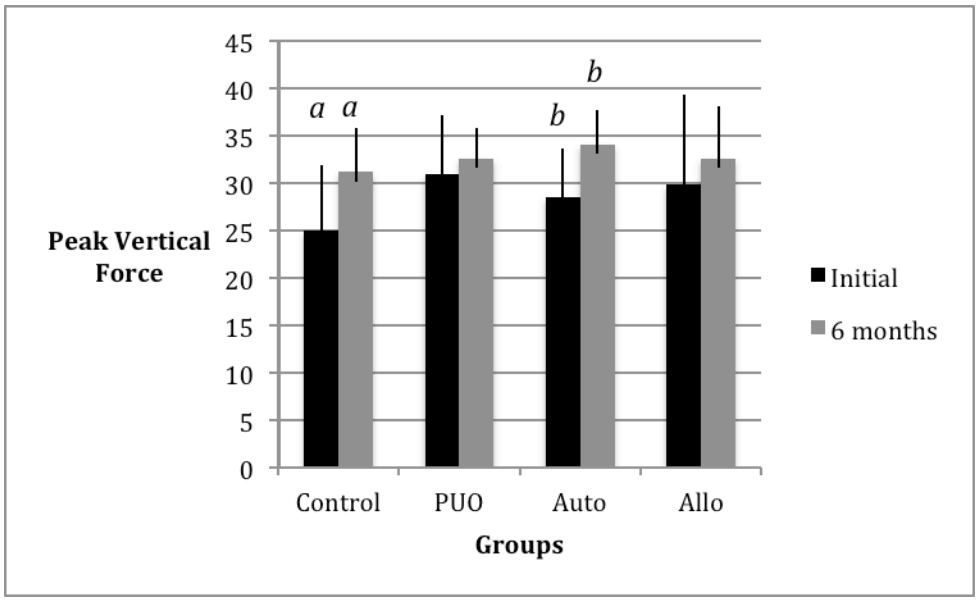


Figure 4.3: Average peak vertical force, corrected for body weight, depicted on the y-axis, with each treatment group depicted on the x-axis. Initial assessment (black) and 6-month assessment (grey) are depicted side by side for each treatment group. Columns labeled with the same letter indicate statistical significance between time points.

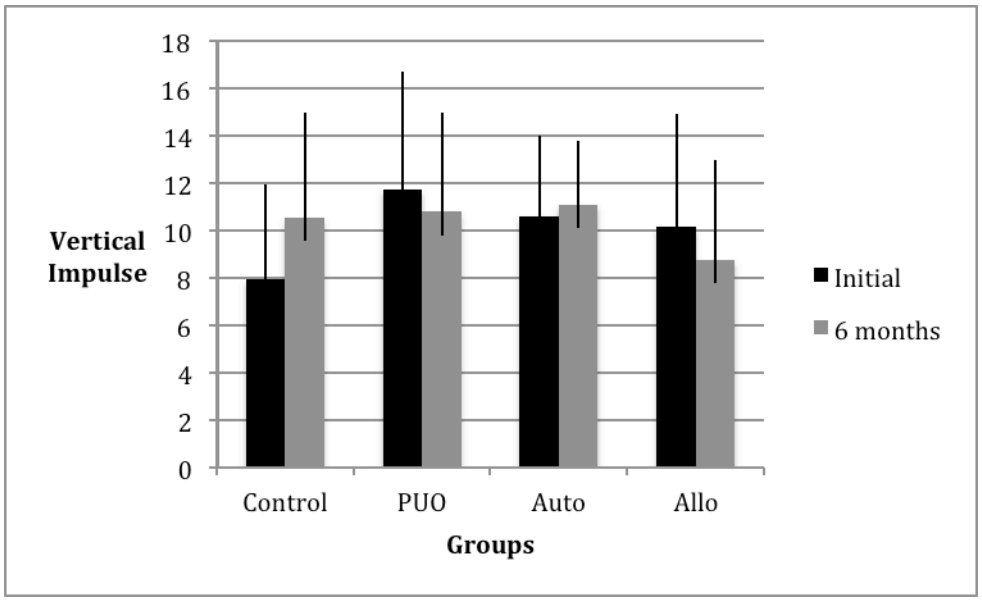


Figure 4.4: Average peak vertical impulse, corrected for body weight, depicted on the y-axis, with each treatment group depicted on the x-axis. Initial assessment (black) and 6-month assessment (grey) are depicted side by side for each treatment group.

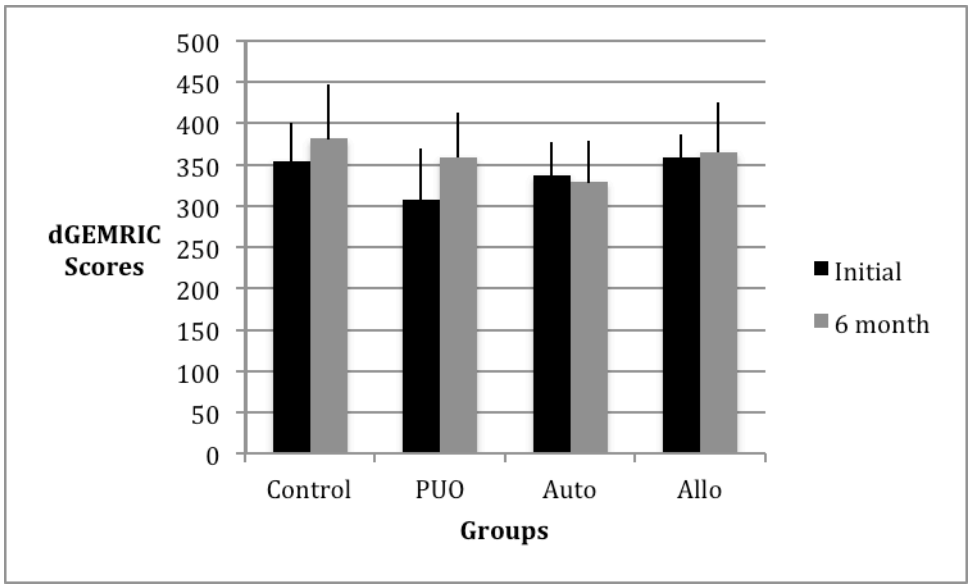


Figure 4.5: Averaged delayed gadolinium enhanced magnetic resonance imaging of cartilage (dGEMRIC) score for each group depicted on the y-axis. Each treatment group is depicted on the x-axis at initial assessment (black) and 6 month recheck (grey) side by side.

Chapter 5

Conclusions

Current practice of canine ASC of osteoarthritis should be questioned by owners and practitioners. The current intra-articular administration of ASCs could result in inconsistent and negative responses in our patients. We suggest flushing a joint with sterile saline prior to administration to provide a less toxic environment for cells, however brief that may be. In vivo response to therapy should be compared between dogs that have had intra-articular administration of cells versus those that have had joints flushed prior to administration. Cell dose should also be assessed in clinical trials. It may be that we are under-dosing our patients, and thus not seeing the anti-inflammatory effect we would expect. If a similar effect and response to therapy is seen across groups, one would expect that there is a significant trophic effect elicited by ASCs prior to cell death.

While we do not understand the pathology of osteoarthritis in enough detail to understand the precise phenotypes that would be most beneficial to provide relief of the disease process, there are factors that are recognized as providing benefit that could be targeted and selected for with culture, expansion and characterization of ASCs prior to administration. This selection of disease specific criterion could greatly enhance response to therapy. There is still much work to be done to investigate if this variability in cell phenotype is most attributable to donor, age, cell source type, or the manipulations we apply after harvest. Availability of easily accessible product should not support neglect of understanding mechanisms of action and enhancing our therapeutic potential.

Allogeneic stem cell therapy should continue to be closely investigated and developed, as this has great potential benefit to understanding the basic scientific questions we should be asking, as well as minimizing variability in product, and thus allowing better assessment of response to therapy. Culture conditions of allogeneic stem cells will need to be critically evaluated and selected according to their specific application. We have demonstrated that cultured cells are phenotypically different than SVF products.

We have demonstrated that allogeneic stem cells can be safely used to treat osteoarthritis, and they may provide improved therapeutic outcome. ASC therapy deserves more thorough, thoughtful and critical assessments in canine veterinary medicine. We are currently blazing the path for this therapy to be used in a likewise manner for humans, and we should take a serious and responsible approach to utilizing it to the best of it's capacity in an appropriate manner. We recommend additional, controlled clinical trials evaluating ASC in an objective manner.

The field of veterinary medicine has a very unique opportunity to lead by example in the regenerative medicine field, benefiting our patients, and hopefully in the future, our friends and family members afflicted with similar diseases. Education of the public and the rest of the veterinary field to proceed cautiously and thoughtfully is critical to maintain this privilege.

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