ADMINISTRATION OF ADIPOSE-DERIVED STROMAL VASCULAR FRACTION AND PLATELET RICH PLASMA IN DOGS WITH COXOFEMORAL OSTEOARTHRITIS.

by

DAVID A. UPCHURCH

B.S., Auburn University, 2006

D.V.M., Auburn University, 2010

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Approved by:

Major Professor
Walter Renberg DVM, MS
Diplomat ACVS

Abstract

Objective: To evaluate the safety and effect of a single simultaneous intra-articular and intravenous injection of autologous adipose-derived stromal vascular fraction (SVF) and platelet rich plasma (PRP) on coxofemoral osteoarthritis (OA) in dogs.

Methods: This was a randomized, double-blind, placebo-controlled prospective pilot trial of simultaneous intra-articular and intravenous SVF and PRP for coxofemoral OA. Dogs with coxofemoral OA causing signs of lameness or discomfort were evaluated by orthopedic exam, visual lameness score, Canine Brief Pain Inventory (CBPI), goniometry, visual analogue scale (VAS), and pressure-sensitive walkway (PSW) at week 0 (baseline), and at 4, 8, 12 and 24 weeks after injection. Joint radiographs were scored at 0 and 24 weeks.

Results: Twenty two client-owned dogs with naturally occurring OA of the coxofemoral joints were enrolled (12 placebo-control, 10 SVF-treated). CBPI pain severity scores were lower in the treatment group at 24 weeks compared to the placebo group (p=0.042). The VAS score for the treatment group was significantly greater at 0 weeks than at 4, 8, or 24 weeks (p<0.05). When dogs with low quartile baseline PVF (25th percentile) were compared, the treatment group had statistically higher PVF at all post-injection time points when compared to the placebo group. After SVF injection, fewer dogs in the treated group were lame compared to the control group.

Clinical Significance: This study is the first to utilize objective data from PSW as an outcome measure for dogs treated with SVF and PRP for coxofemoral OA. No adverse events were noted. Improvements in some measured parameters in the treated dogs compared to those in the placebo group.

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List of Abbreviations

OA – Osteoarthritis

IL-1 – Interleukin 1

IL-6 – Interleukin 6

TNF-α – Tumor Necrosis Factor Alpha

ECM – Extracellular Matrix

MMP – Matrix Metalloproteinase

MMP-1 – Matrix Metalloproteinase 1

MMP-2 – Matrix Metalloproteinase 2

MMP-13 – Matrix Metalloproteinase 13

ADAMTS – A Disintegrin-like and Metalloproteinase with Thrombospondin Type 1 Motifs

ADAMTS-5 – A Disintegrin-like and Metalloproteinase with Thrombospondin Type 1 Motifs 5

PGE_2 – Prostaglandin E_2

IGF-1 – Insulin-like Growth Factor 1

IGF-2 – Insulin-like Growth Factor 2

TGF-β – Transforming Growth Factor Beta

MSC – Mesenchymal Stromal Cell

AMSC – Adipose Derived Mesenchymal Stromal Cells

SVF – Stromal Vascular Fraction

PTE – Pulmonary Thromboembolism

IL-10 – Interleukin 10

PVF – Peak Vertical Force

PSW – Pressure Sensitive Walkway

NSAID – Non-steroidal Anti-inflammatory Drug

CBPI – Canine Brief Pain Inventory

PS – Pain Severity

PI – Pain Interference

ROM – Range of Motion

VAS – Visual Analogue Scale

VI – Vertical Impulse

PRP – Platelet Rich Plasma

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Dedication

I would like to dedicate this thesis to my wife Melissa and daughter Ember who have supported me throughout my academic and professional career. Without their support and dedication to helping me follow my goals and dreams, I would never have been able to accomplish any of what you read below. I owe them more than I can ever possibly repay.

Chapter 1 - Mesenchymal Stromal Cell Therapy Literature Review

Pathogenesis of Osteoarthritis and Hip Dysplasia

Osteoarthritis (OA), also known as degenerative joint disease, is a pathologic condition of diarthrodial joints that includes aberrant repair and degradation of the articular cartilage. Joint pathology associated with OA also includes subchondral bone sclerosis, osteophytosis and synovitis (1). Numerous underlying causes can exist that lead to the development of OA. In human medicine, primary degenerative joint disease develops due to chronic stress and senescence of articular cartilage in elderly individuals (2). Secondary degenerative joint disease, which is far more common in small animal species, is defined as OA secondary to a variety of *precipitating* factors included, but not limited to chronic joint instability, osteochondrosis, trauma, or ligamentous damage (2-4). *Predisposing* factors, such as genetic predisposition, age, higher birth weight, obesity, and gender status have also been shown to contribute to the development of OA (5-9). Depending on the precipitating and predisposing factors initial pathology of degenerative joint disease can vary from structural damage to synovial inflammation depending on the underlying factors. Regardless of the initial pathology, altered stresses on the articular cartilage will lead to chondrocyte damage and release of pro-inflammatory mediators which cause synovial inflammation as well damage to collagen and aggrecan molecules that form the structural framework of cartilage. The result is a loss of cartilage resiliency and further cartilage damage that ultimately leads to cartilage loss, subchondral sclerosis, and osteophyte formation. Initially, chondrocytes compensate for this destruction through increased metabolic activity and proliferation, but as joint pathology

progresses, the chondrocytes reparative processes are offset by the catabolic processes in the joint and progressive cartilage destruction results (1, 10).

In veterinary studies, it has been estimated that 20% of adult dogs and 60% of adult cats are affected with OA (11). Hip dysplasia is the most common orthopedic condition in the dog (12, 13). In the hip joint, it is widely believed that underling hip laxity predisposes to the development of OA. While the exact mechanism by which laxity leads to OA is unknown, one proposed mechanism is that passive laxity of the joint occurring during the gait cycle leads to subluxation of the femoral head and abnormal force distribution across the joint resulting in premature damage to articular cartilage (13).

The Role of Inflammatory Mediators, Cytokines and Growth Factors in Osteoarthritis

Regardless of the factors that cause OA in a joint, cartilage damage leads to an up-regulation of pro-inflammatory cytokines. Interleukins 1, 6, and tumor necrosis factor-alpha (IL-1, IL-6, and TNF-α) are considered to be the main pro-inflammatory cytokines active in OA. All three are produced by chondrocytes, and IL-1 and TNF-α can also be produced by mononuclear cells, osteoblasts, and synoviocytes (1, 4). While a low level of all three of these cytokines are found in normal joints, cartilage damage up-regulates the production of these inflammatory mediators and increased levels can be detected in the synovial fluid, synovium, subchondral bone and cartilage (14-16). In cell culture studies, IL-1 alters the structure of articular cartilage by reducing the production of type II collagen and aggrecan (17-19). TNF-α suppresses the synthesis of proteoglycans (20). All three pro-inflammatory mediators (IL-1, IL-6, and TNF-α) bind to chondrocytes and stimulate the production of several proteolytic enzymes such as the matrix metalloproteinases (MMPs), MMP-1, MMP-3, and MMP-13, which are key regulators in the progression of cartilage destruction (21-23). Increasing evidence also implicates a family of proteins known as ADAMTS (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs), especially ADAMTS-5, in progressive cartilage destruction. This protease is produced by chondrocytes and upregulated by IL-1 and TNF-α (24).

Other chemical mediators that are upregulated in OA include interleukins 15, 17, 18, and 21 as well as leukemia inhibitory factor, prostaglandin E_2 (PGE_2) and nitric oxide (4). These compounds have an additive effect on decreased ECM synthesis and progressive cartilage destruction. Nitric oxide can also directly promote chondrocyte apoptosis, likely via direct effects on mitochondrial metabolism (25).

The pro-inflammatory cytokines released from the cartilage also have an effect on other cells within the joint. Macrophages and lymphocytes are often recruited into the synovium and triggered by cytokines to release collagenase and other hydrolytic enzymes (26). Macrophages also secrete IL-1 and TNF-α, furthering the inflammatory cascade and potentiating a catabolic state (4).

The pro-inflammatory molecules in normal diarthrodial joints are balanced by anti-inflammatory molecules that promote anabolic functions including insulin-like growth factor 1 and 2 (IGF-1, IGF 2) and transforming growth factor – beta (TGF-β). These molecules stimulate aggrecan and collagen synthesis by chondrocytes. In the early stages of joint disease, these molecules may also be up-regulated. With the development of end stage OA, however, expression of IGF-1, IGF-2 and TGF-β expression are all down-regulated by as yet undefined mechanisms. This leads to an imbalance in the pro-inflammatory and anti-inflammatory cascades favoring catabolic processes (27).

Stem Cells, Stromal Cells and Stromal Vascular Fraction

Stem cell therapy is a regenerative therapy that is aimed at utilizing progenitor cells to modulate inflammation and repair damage in living tissues. Most of the pioneering research in stem cell therapy was performed in the 1960s by E. Donnal Thomas and focused on replacement of hematopoietic cells after full body irradiation in humans and animal models (28). A strict definition of stem cells has not been agreed upon, but a number of properties are considered characteristic of stem cells, including extensive self-renewal capacity, the ability to exist in a mitotically quiescent form, and the ability to clonally regenerate different cell types that constitute the tissue in which they exist (29). In the adult organism, a population of mesenchymal progenitor cells exists that are capable of differentiating into chondrogenic, adipogenic and osteogenic lineages (30-31). While many authors call these cells mesenchymal stem cells, the ability of these cells to generate functional progeny *in vivo* has not been demonstrated, so a more appropriate term is mesenchymal stromal cells (MSCs).

MSCs have been isolated from bone marrow, adipose tissue, umbilical cord tissue, muscle, synovium, periosteum, dermis, deciduous teeth, and bone (30, 32, 33). While species and site differences have been reported in the differentiation potential of various sources of MSCs, all of the sources contain MSCs capable of chondrogenic, osteogenic, and adipogenic differentiation (32, 34, 35). Adipose tissue and bone marrow are the two most easily accessible sources of MSCs. In human medicine, adipose derived mesenchymal stromal cells (AMSCs) contain 500 times more MSCs per milliliter than bone marrow, making it an attractive source of these cells (36).

Unfortunately, obtaining a pure sample of AMSCs requires *in vitro* culturing on plastic culture plates, as MSCs are plastic adherent (31). Further purification occurs through a series of

cell washes and culture expansion with the endpoint being a population of adherent AMSCs.   In addition to requiring weeks to months to accomplish, serial expansion of AMSCs *in vitro* may lead to loss of differentiation, contamination, or neoplastic transformation of the cells (30, 37). Because of this, research has been conducted into the use of the cellular fraction of adipose tissue, known as the stromal vascular fraction (SVF). While no consensus has been obtained regarding a specific procedure, adipose tissue is generally mechanically minced and then digested by the addition of collagenase, dispase, trypsin or related enzymes at 37°C for 30 minutes to one hour. The enzymes are then neutralized and the released cells (known as the SVF) are separated from the fat via centrifugation. This procedure generally takes 4-5 hours and requires no *in vitro* culture or expansion (38).

SVF was first described in the 1960s by Hollenberg, and it was discovered in 2001 that it contained a reservoir of MSCs (39, 40). In addition to MSCs, SVF contains a variety of other cell types including endothelial precursor cells, monocytes, macrophages, T-regulatory cells, pericytes, mast cells, preadipocytes, fibroblasts and smooth muscle cells (30, 37). In fact, only $17 \pm 3.2\%$ of SVF cells were found to be MSCs in one canine study (41).

Proposed Mechanisms of Action of Mesenchymal Stromal Cells and Stromal Vascular Fraction

Mesenchymal stromal cells, which can be utilized either by themselves or in SVF therapy, are proposed to exert a therapeutic effect on OA via a number of mechanisms including the replacement of damaged cells, enhancing the replication of tissue cells, anti-inflammatory and immunomodulatory effects, anti-apoptotic effects and pro-angiogenic effects (30, 42).

MSCs from both adipose and bone marrow have been fluorescently labeled and injected into host tissue to detect their ability to engraft into the tissue. Studies have shown engraftment of these fluorescently labelled cells into synovium, meniscal cartilage and intra-articular fat after intra-articular injection (43). Engraftment has been shown in spinal cord (44), myocardium (45) skeletal muscle (46), and urinary bladder smooth muscle (47) after direct injection into these tissues. Engraftment of MSCs into the liver of humans suffering from graft-vs-host disease was evident after intravenous injection (48). Finally, the use of a beta tricalcium phosphate/ hydroxyapatite scaffold inoculated with MSCs showed engraftment of MSCs into the adjacent bone (49). Most of these studies only evaluated engraftment, but a few also showed differentiation of MSCs into target tissue cells (45, 47).

Engraftment and differentiation into target tissues is only one proposed mechanism by which MSCs exert beneficial effects. MSCs are also anti-inflammatory and immunomodulatory. It has been shown that MSCs secrete IL-1 receptor antagonist, which binds to the receptors for IL-1 and blocks IL-1 mediated inflammation (50). MSCs have been shown to inhibit T-cell, B-cell and natural killer cell proliferation and activation as well as to down-regulate major histocompatibility complex II expression on inflammatory cells. The exact mechanism of action of these effects in unknown, but they potentially occur due to MSCs decreasing T-cell homing to

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target tissues, and suppression of IL-1, TNF, and interferon gamma, as well as production of the anti-inflammatory cytokine interleukin 10 (IL-10) (33,48,51). MSCs have been shown to suppress dendritic cell maturation (which leads to generation of immunomodulatory T-regulatory cells) and suppress macrophage activation (52, 53).

While MSC can be delivered directly into a target tissue, it is also possible to inject them intravenously. While the mechanism is not completely understood, MSC appear to be able to home to areas of inflammation. In a study evaluating intravenous MSC injection in mice, non-arthritic mice had MSC identified in the lungs and liver at 14 days post-injection, whereas arthritic mice also had MSC isolated from the affected joints (54).

When humans MSCs were cultured for 72 hours in hypoxic conditions (1% oxygen), they secreted five-fold greater amounts of vascular endothelial growth factor (which functions to stimulate angiogenesis) when compared to MSCs cultured in normoxic conditions (21% oxygen) (46). In the same study, endothelial cells cultured in media from hypoxic MSCs had decreased apoptosis, indicating the presence of anti-apoptotic secretions from the MSCs. This combination of anti-inflammatory, pro-angiogenic and anti-apoptotic effects, while not completely understood, provides the basis behind the use of MSCs for treatment of OA, as the mechanisms of action of MSCs seem to inhibit pro-inflammatory cytokines (IL-1) present in osteoarthritic joints.

Although MSC are considered immunologically privileged, evidence exists that MSC transplanted between major histocompatibility complex mismatched individuals may lead to alterations in the immunomodulatory effects of the MSCs. In a mouse model of OA, injection of major histocompatibility complex mismatched allogeneic MSCs not only had less

immunomodulatory effects *in vitro* compared to MHC matched cells but also had a negative effect on progression of OA *in vivo*, characterized by increased concentrations of TNF-α (55).

SVF contains MSCs as well as a variety of other cell populations that may have additive effects on the anti-inflammatory and pro-angiogenic properties of the MSCs. Endothelial precursor cells have been identified in human SVF and these cells were capable of inducing angiogenesis in a mouse model of hindlimb ischemia (56). Macrophages isolated from human SVF were found to exhibit predominantly an M2 phenotype characterized by increased secretion of IL-10 and IL-1 receptor antagonist (57-59). T-cells found in the SVF are exposed to high concentrations of IL-10 (produced by macrophages and MSCs). T-regulatory cells themselves help to maintain the M2 phenotype of macrophages and to act in an immunosuppressive capacity (37). Because of the combined effect of MSCs and these other cell types found in SVF, it is possible that SVF therapy leads to more profound anti-inflammatory and immunomodulatory effects than MSCs themselves.

The safety of MSC treatment has been called into question, with concerns including increased patient susceptibility to infection due to the immunomodulatory effects of the MSC, embolism of the cells, and acute or chronic immune reactions to the cells themselves (60). One meta-analysis of 36 human studies that utilized intravenous MSC therapy on a total of 1012 patients found that there was no increased risk of adverse effects with MSC therapy, with the exception of an increased risk of transient fever (OR 16.82, 95% CI 5.33–53.10) (61). A more recent article found that after mice received an intravenous injection of 1.5×10^5 adipose derived MSCs there was an 85% mortality rate within 24 hours due to pulmonary thromboembolism (PTE). It was discovered that the MSCs exerted a pro-coagulant effect via stimulation of the extrinsic clotting cascade (61, 62). The authors of that study speculated that the MSCs expressed

tissue factor which stimulated the clotting cascade. Similar pro-coagulation effects were found

in vitro when human adipose-derived MSCs were exposed to allogenic human blood or plasma

(63). One human case of pulmonary thromboembolism associated with MSC therapy has been

reported (64), and there have been at least two human fatalities associated with MSC therapy

(65). The safety of MSCs delivered via alternative routes and the safety of SVF have not yet

been assessed.

Human and Veterinary Studies of Stromal Vascular Fraction Therapy

While numerous case series and clinical trials exist to evaluate the therapeutic use of MSCs, the use of SVF in human medicine is currently a developing field. Currently, SVF is not regulated by the FDA, but rather by state medical boards and professional societies (66). Nonetheless, although a number of pre-clinical trials and case reports have been published, few prospective clinical trials have been completed. Pre-clinical trials have been conducted in laboratory animals on the use of SVF for treatment of myocardial infarction, spinal cord trauma, inflammatory bowel syndrome, diabetes mellitus, tendonitis, urinary incontinence, hindlimb ischemia and other diseases (67-73).

Human clinical case reports have been published using SVF to treat irradiation fibrosis, Crohn's disease, multiple sclerosis, breast augmentation, and craniofacial trauma (73-78). Only one human study to date has been conducted on the use of SVF for treatment of OA (79). In this non-blinded prospective study a total of 1856 arthritic joints from 1128 individuals were injected with a single intra-articular dose of autologous SVF. Patients were evaluated at 3, 6 and 12 months after the injection and were assigned an outcome score at each time point based on pain, non-steroid analgesic usage, limping, extent of joint movement, and stiffness evaluation. No serious side effects, systemic infection, or cancer was associated with SVF cell therapy. SVF treatment resulted in at least 50% improvement in 80.6% of patients at 3 months and 91.0% of patients at 12 months. Only 0.9% of patients were considered to be non-responders, with the rest showing <50% improvement. It is important to note that this study was not placebo-controlled or blinded, and had no objective outcome measures.

There are four studies that have evaluated the effect of SVF on OA in veterinary species. In one double-blinded randomized controlled trial, 18 animals with bilateral coxofemoral OA

were treated with a single intra-articular injection of autologous SVF or with a placebo control. Outcome was assessed at 30, 60 and 90 days post-treatment and outcome measures included lameness evaluation, pain, range of motion, and owner perceived changes in functional disability. A significant decrease in pain, lameness and significant increase in range of motion was observed from baseline to all three time-points in the treatment group. Mean effect sizes were significant (greater than 0.8) for pain (1.57), lameness at a trot (1.36), range of motion (1.45), and a composite score (1.34). No significant change was observed in owner-perceived functional disability, and no significant changes occurred in any parameters in the control group (80).

A non-blinded study conducted by the same group assessed 14 dogs with elbow OA treated with a single intra-articular injection of autologous SVF. Outcome measures were the same as the study above, but follow-up assessments were performed at 30, 60, 90, and 180 days after treatment. Lameness score, range of motion, and owner perceived functional disability were significantly improved at all time-points compared to baseline, but pain on manipulation did not significantly improve. This study did not use a placebo control, and, as a result, evaluators were not blinded to treatments (81).

A prospective, blinded, placebo-controlled study was conducted to directly compare bone marrow derived MSCs to SVF using an experimental equine model of OA. Twenty-four horses each had an osteochondral defect surgically created in one of their middle carpal joints. Fourteen days later, they were given an intra-articular injection of either autologous SVF, autologous bone marrow derived MSCs or a saline placebo into both the osteoarthritic carpal joint as well as the contralateral unaltered carpal joint. Pain, range of motion, and effusion of the joints were assessed every two weeks, synovial fluid analysis was conducted weekly, and radiographs were

conducted at 7, 14, and 70 days after surgery. Finally, gross necropsy and histopathological examination of the joints were conducted at 70 days after surgery. While significant differences were noted between operated and non-operated limbs, no changes were observed between treatment groups in regards to pain score, effusion score, or radiographic assessment. Joint fluid analysis showed a significant decrease in the number of lymphocytes in joints treated with SVF, and osteoarthritic joints with SVF also had a significant increase in TNF-α compared with normal joints treated with SVF. No other cytological or histology differences were found between different treatment groups. The authors concluded no overt treatment benefit of either MSCs or SVF (82).

Finally, a non-blinded study was conducted in nine dogs with coxofemoral OA. In this study four dogs were treated with autologous SVF and five dogs were treated with allogenic AMSCs. All dogs were given a single set of injections into acupuncture points bladder 54, gallbladder 29, and gallbladder 30. These dogs were subjectively assessed at approximately 7, 15, and 30 days post-injection for range of motion, pain and owner perceived functional disability assessment. All outcome measures were reported to be improved by day 15 in 4/4 dogs treated with SVF and 3/5 dogs treated with AMSCs (83).

In summary, it is important to note that all of the current research on the use of SVF for treatment of OA uses outcome measures that are subjective in nature. Only two out of the four veterinary studies utilized a placebo group and only one of the four utilized histopathological and radiographic findings. This latter study found no evidence of improved outcome with the use of SVF compared to placebo. As of the time of publication, there have been no prospective, randomized clinical trials with objective outcome measures assessing the use of SVF for the treatment of OA.

Conclusions

Osteoarthritis is a disease that is present in a large subset of both veterinary and human patients, and it can be debilitating. The pathophysiology of OA is not completely understood, but it is known that osteoarthritic joints suffer from an imbalance between pro-inflammatory and anti-inflammatory cytokines that leads to ongoing cartilage destruction and joint catabolism. MSCs and other components of SVF including endothelial precursor cells, type M2 macrophages, T-regulatory cells, microvesicles, and other secreted factors directly inhibit some of the pro-inflammatory mediators of OA. As a result, SVF injections into arthritic joints warrant consideration as a treatment option for this disease. Current clinical evidence is inconclusive lacks objective outcome measures, and consists of few studies that show conflicting results about the benefit of SVF. Further research conducted using fully powered, appropriately designed, randomized control trials with larger numbers of patients, as well as objective outcome measures is required to obtain a more comprehensive picture of the safety profile and efficacy prior to FDA approval of SVF therapy for OA.

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Chapter 2 - Administration of Adipose-derived Stromal Vascular Fraction and Platelet Rich Plasma in Dogs with Coxofemoral Osteoarthritis.

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"Administration of Adipose-derived Stromal Vascular Fraction and Platelet Rich Plasma in Dogs with Coxofemoral Osteoarthritis. "

David Upchurch, DVM[1]

Walter Renberg, DVM, MS, DACVS[1]

James Roush, DVM, MS, DACVS[1]

George Milliken, PhD[3]

Mark L. Weiss, Ph.D[2]

[1]Department of Clinical Science, Kansas State University, Manhattan, KS, USA

[2]Department of Anatomy and Physiology, Kansas State University, Manhattan, KS, USA

[3]Milliken Associates, Inc, 1401 Deep Creek Lane, Manhattan, KS, USA

Abstract

Objective: To evaluate the safety and effect of a single simultaneous intra-articular and intravenous injection of autologous adipose-derived stromal vascular fraction (SVF) and platelet rich plasma (PRP) on coxofemoral osteoarthritis (OA) in dogs.

Methods: This was a randomized, double-blind, placebo-controlled prospective pilot trial of simultaneous intra-articular and intravenous SVF and PRP for coxofemoral OA. Dogs with coxofemoral OA causing signs of lameness or discomfort were evaluated by orthopedic exam, visual lameness score, Canine Brief Pain Inventory (CBPI), goniometry, visual analogue scale (VAS), and pressure-sensitive walkway (PSW) at week 0 (baseline), and at 4, 8, 12 and 24 weeks after injection. Joint radiographs were scored at 0 and 24 weeks.

Results: Twenty two client-owned dogs with naturally occurring OA of the coxofemoral joints were enrolled (12 placebo-control, 10 SVF-treated). CBPI pain severity scores were lower in the treatment group at 24 weeks compared to the placebo group (p=0.042). The VAS score for the treatment group was significantly greater at 0 weeks than at 4, 8, or 24 weeks (p<0.05). When dogs with low quartile baseline PVF (25th percentile) were compared, the treatment group had statistically higher PVF at all post-injection time points when compared to the placebo group. After SVF injection, fewer dogs in the treated group were lame compared to the control group.

Clinical Significance: This study is the first to utilize objective data from PSW as an outcome measure for dogs treated with SVF and PRP for coxofemoral OA. No adverse events were noted. Improvements in some measured parameters in the treated dogs compared to those in the placebo group.

Introduction

Hip dysplasia is one of the most common orthopedic abnormalities in dogs, with some breeds having up to a 40% incidence (1-4). Current treatment strategies for osteoarthritis (OA) include decreasing the magnitude of forces on osteoarthritic joints via weight loss and exercise, palliating joint discomfort by hip replacement, or the use of medications that modulate disease signs. Commonly used pharmaceutical agents to treat OA include non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit prostaglandin E_2 (PGE_2) via cyclooxygenase inhibition, and nutraceuticals, such as glucosamine and hyaluronan, which may function via anti-inflammatory activity (5). These drugs do not target the majority of pro-inflammatory mediators, and they are unable to stop the catabolic state present in an osteoarthritic joint.

An ideal treatment modality would halt or reverse the inflammatory cascade that causes OA, and mesenchymal stromal cell (MSC) therapy can theoretically achieve that goal. MSCs have been shown to engraft into host tissues and differentiate into target cells. A proposed mechanism of action of MSCs in OA therapy is via immunomodulation and reversal of the pro-inflammatory cascade (6, 7). In vitro, MSC's mechanism of action is both contact-mediated (smaller effect) via an unknown mechanism and contact-independent (larger effect) which is mediated by immune modifying chemicals. For example, MSCs secrete interlukin-1 (IL-1) receptor antagonist, a potent inhibitor of IL-1 (8). It has been theorized that they also inhibit tumor necrosis factor- α (TNF-α, and produce the anti-inflammatory cytokine interleukin 10(IL-10) (8-10). MSCs inhibit activated T-cell, B-cell and natural killer cell proliferation and down-regulate major histocompatibility complex II expression on inflammatory cells (9, 10). MSCs can suppress dendritic cell maturation (which leads to generation of T-regulatory cells) and suppress macrophage activation (11, 12).

Adipose tissue is easily harvested from most dogs and is a rich source of MSCs. In humans, adipose contains 500 times more MSCs per milliliter than bone marrow, the other commonly utilized source of autologous MSCs (13). In order to obtain MSCs from adipose tissue, a series of cell isolation steps followed by expansion of MSCs *in vitro* is needed (14). This requires weeks to accomplish, and long term expansion of MSCs *in vitro* may lead to loss of differentiation potential, contamination, DNA damage, or neoplastic transformation (15, 16). Furthermore*, in vitro* expansion of cells requires Good Cell Manufacturing Processing protocols in a Food and Drug Administration (FDA) inspected facility to comply with FDA manufacturing requirements (Industry Guidance #218 dated June 2015). Because of these restrictions, research has been conducted into the use of the autologous stromal vascular fraction (SVF) which can be harvested in a fashion that meets the "minimal manipulation criteria" established by the FDA. While techniques vary for SVF isolation, adipose tissue is minced then digested by collagenase, dispase, trypsin, or related enzymes in a procedure that takes several hours and requires no *in vitro* culture or expansion (17). In addition to MSCs, SVF contains other cell types including endothelial precursor cells, monocytes, macrophages, T-regulatory cells, pericytes, mast cells, preadipocytes, fibroblasts and smooth muscle cells (15, 16). These other cell types may enhance the beneficial effects of the MSCs. For example, endothelial precursor cells may secrete the pro-angiogenic mediator, vascular endothelial growth factor (VEGF); M2 macrophages, may secrete IL-10 and IL-1 receptor antagonist; and T-regulatory cells may help to maintain the M2 phenotype of macrophages and act in an immunosuppressive capacity (16, 18-21). MSC injected intravenously have been shown to home to areas of inflammation, including joints, where they can then exert local anti-inflammatory and immunomodulatory effects (22).

Previous *in vitro* studies have shown that injection of MSCs into joints with experimentally induced OA can decrease cartilage destruction, osteophyte formation and subchondral sclerosis, and even lead to regeneration of meniscal and articular cartilage (23-26). Five studies reported improved clinical outcomes when MSC are injected intra-articularly into the joints of animals affected by experimentally-induced osteoarthritis (27-31). Several veterinary clinical studies used subjective measures such as owner-perceived functional disability, and veterinary lameness and pain evaluation as outcome measures; three studies found improvement in some or all of these criteria for up to six months after treatment (27-29). Two veterinary clinical studies utilized quantitative outcomes measures (a pressure-sensitive walkway) to evaluate dogs after MSC treatment of hip OA. These studies found an improvement in peak vertical force (PVF) of the more lame limb of treated dogs at three months after a single treatment (30, 31).

Autologous platelet rich plasma (PRP) has been used as a treatment of OA (32, 33), but it can also be co-administered with MSC or SVF. PRP contains several growth factors including VEGF, platelet derived growth factor, fibroblast growth factor 2 and transforming growth factor beta which can enhance regenerative processes and promote healing of intra-articular structures (32, 33). PRP co-administered with adipose-derived MSCs led to enhanced proliferation of the MSCs as well as improved joint function and cartilage regeneration compared to MSCs alone in a mouse model of OA (33).

SVF therapy has become increasingly available to veterinary medicine clinics in the United States via several commercially available platforms and procedures. Questions remain regarding efficacy and safety because few studies with low numbers of client-owned animals have evaluated SVF as an OA treatment (34-38). Studies that evaluate SVF therapy have shown

improvement in subjective outcome measures for three to six months after a single treatment (39-41). Only one study included radiographic and histopathological data (42), and this study concluded that SVF therapy had no benefit over either MSC therapy or saline placebo for treatment of experimentally-induced equine carpal OA. At the time of submission of this article, no randomized, placebo-controlled, double-blind study has evaluated the use of autologous SVF and PRP for the treatment of canine hip OA with quantitative outcome measures (e.g. using PSW analysis).

We performed a randomized, double-blind, placebo-controlled pilot to evaluate the safety and efficacy of autologous intra-articular and intravenous SVF and PRP for treatment of naturally-occurring OA of the hips in dogs. Collected outcome measures were veterinary lameness exam score (39), a previously validated owner survey of pain (43, 44), goniometry (45), a visual analogue scale (VAS) completed by the veterinarian (46), and ground forces data obtained via a pressure sensing walkway (PSW). We hypothesized that dogs receiving SVF/PRP injections would improve veterinary lameness scores, increase range-of-motion in the injected joint, decreased pain scores, and improve weight bearing demonstrated on PSW analysis of the treated limb compared to baseline measures. We also hypothesized that the SVF/PRP injections would be safe, as defined by a lack of adverse effects upon infusion and a lack of increased incidence of adverse events over the six month study period.

Materials and Methods

The study population consisted of client-owned dogs recruited from pet owners through the use of electronic mail alerts, as well as online, print, and radio advertisements. Costs associated with lameness evaluation and treatments were paid by grant funds and owners received no financial incentive to participate. This study was approved by the Kanas State University Institutional Animal Care and Use Committee (IACUC registry number 3303). This study was reviewed by FDA as an Investigation of New Animal Drug (INAD registry number 12457). Dog owners signed an informed consent.

Dogs were evaluated at baseline (week 0, prior to adipose collection, SVF isolation and joint injection), and at 4, 8, 12 and 24 weeks after joint injection. Experimental group allocations were randomized with a random-number table and were allocated in a 1:1 ratio to treatment or placebo groups prior to study enrollment. In order to be eligible for the study, dogs were required to have a body weight > 15kg; have either a history of lameness or dysfunction attributable to OA as reported by the owner (decreased activity level, pain, inability to rise from standing); demonstrate either a 5% or greater difference in PVF measured as a percentage of body weight (kg) between the two hind limbs (to establish one leg as clinically worse than the other) or a PVF less than 34% combined of body weight in both hindlimbs on a pressure sensitive walkway (PSW)[a]; and have radiographic evidence of OA of the coxofemoral joint of the lame limb. Prior to enrollment, dogs were permitted to be on non-steroidal anti-inflammatories (NSAIDs), symptom modifying OA drugs, therapeutic diets or other analgesics except for corticosteroids, and no changes in medications or supplements were permitted for two weeks prior to enrollment or during the 24 week study period. Exclusion criteria included dogs with unsuitable temperament, historical or physical exam evidence of other hindlimb pathology (stifle or hock

pathology, neurologic gait disorders), changes to analgesic medications within 2 weeks of study enrollment or during the study, or evidence of systemic disease on physical examination or bloodwork.

Initial Evaluation

Prior to enrollment in the study, all candidate dogs were examined by one of the investigators who was blinded to the experimental group allocation (DU). Initial evaluation included determination of patient age, sex, breed, the limb or limbs affected, the duration of lameness, history of prior orthopedic surgery, and the type and duration of current pain medications or supplements. Body weight (kg) and body condition score (BCS) on a 1-9 scale were recorded by a single observer (DU). A complete orthopedic and neurologic examination and visual veterinary lameness exam were performed with a lameness grade of 1-6 assigned by a single observer (DU) as previously reported and described in Appendix 1 (39).

Owners were also blinded to experimental group allocation of their pets. Owners were given the Canine Brief Pain Inventory (CBPI) with the same individual required to complete the survey during each evaluation. The CBPI is a previously validated two-part owner questionnaire evaluating both pain severity (PS, questions 1-4) and pain interference (PI, questions 5-10) associated with daily activities (43, 44).

Goniometry was performed by a single observer (DU) using a two-arm metal goniometer[b] with 1° increments as previously reported (45). This procedure was repeated for the contralateral coxofemoral joint. A visual analog pain scale (VAS) was completed by the investigator (DU) after the orthopedic assessment as previously described (46).

Following examination, the dog was walked across a Tekscan PSW[a] by one of two handlers. Prior to data collection, the dogs were walked across the PSW until they appeared

comfortable and acclimated to the testing room. A valid trial was defined as one in which the dog walked at a steady pace and in a straight line along the entire length of the PSW and in which each foot strike fell within the recording area of the mat. Five valid trials were obtained for each dog. Data from these five trials were analyzed using system-specific software[c] with stance time, stride velocity, PVF, vertical impulse (VI), and maximum peak pressure recorded.

Following PSW evaluation, dogs meeting the inclusion criteria were sedated with hydromorphone[d] (0.08mg/kg intravenously) and acepromazine[e] (0.02mg/kg intravenously) to obtain lateral and ventrodorsal extended- leg pelvic radiographs. A complete blood count and serum chemistry analysis were performed on all patients. Enrollment was completed if radiographs confirmed OA of the affected joint and no significant abnormalities were detected on bloodwork.

Adipose Tissue Collection

Within three months of initial evaluation, adipose tissue harvesting was performed for each patient. Pre-operative sedation was performed with acepromazine[e] (0.01-0.03mg/kg intravenously) and hydromorphone[d] (0.08-0.1mg/kg intravenously). After induction of general anesthesia (propofol[f], 2-8 mg/kg), the animals were placed in dorsal recumbency. Eighteen milliliters of blood were collected for preparation of PRP. The ventral abdomen was clipped and aseptically prepared for surgery. A 5 cm surgical incision was made on midline just cranial to the umbilicus, extended though the underlying linea alba, and a minimum of 40 grams of falciform adipose tissue was harvested. The adipose tissue was placed in a sterile plastic container. The incision was closed routinely and the dogs were allowed to recover from anesthesia and released to the owners after the intra-articular injection later that day. Post-operative analgesia was administered (codeine sulfate[g], 1-2mg/kg by mouth every eight hours)

for the first 72 hours after surgery. Operative time, anesthesia time and post-operative complications were recorded.

Adipose Tissue Processing

After fat collection, a standardized quantity of fat (enough to fill a 40mL container) was processed for each animal. The samples were processed immediately by a trained technician using a commercial protocol[h] to produce SVF and PRP. Following SVF processing, all samples were suspended in autologous PRP and exposed to a light source for 20 min. A cell viability counter[i] was used to count the number of live nucleated cells per ml and to determine a live/dead ratio. An aliquot was used to confirm MSC content by expansion following plating on tissue culture treated plastic. SVF was plated in 20% fetal bovine serum containing Dulbecco's modified eagle medium at a density of 30,000 live cells per cm^2 and placed into 95% humidity, 5% CO_2, and 37 ˚C incubator to demonstrate cell attachment and expansion consistent with MSC phenotype.

Injection of Stromal Vascular Fraction

Based on the randomization protocol, SVF or placebo (sterile saline[j]) was administered by intra-articular injection the same day as adipose tissue harvesting. Using the data obtained from the baseline PSW trials, the limb that had a lower PVF was selected to receive an intra-articular injection. If both limbs had the same PVF, one limb was selected at random for injection. Dogs were sedated as needed with acepromazine[e] (0.01-0.03mg/kg intravenously) and hydromorphone[d] (0.08-0.1mg/kg intravenously) and an area overlying the joint to be injected was clipped and aseptically prepared. One of the clinical investigators who was blinded to the experimental group allocation (WR) performed an arthrocentesis of the hip joint. Synovial fluid

was aspirated and 0.2-5mL was removed from the joint if possible, after which 0.5mL of the

SVF/ PRP (treatment group) or 0.5mL of sterile saline[j] (placebo group) was injected into the

joint and 0.5mL of the SVF/ PRP or 0.5mL of sterile saline was injected intravenously into a

cephalic vein.

Follow-up Evaluations

All dogs were reassessed at 4, 8, 12, and 24 weeks after injection. At each time point one

investigator (DU) evaluated the animal. Dogs were evaluated as previously described, and data

was recorded for body weight, body condition score, lameness score (Appendix A), and ROM of

both coxofemoral joints. At all time periods, the investigator (DU) filled out a VAS after

completing an orthopedic and neurologic examination and the ground forces data were collected.

At each reassessment the owner was asked to fill out a CBPI, with the same individual answering

the questions at each time point.

Pelvic radiographs were repeated at the 24-week reassessment following examination and

PSW evaluation under the same protocols described above. Radiographs from weeks 0 and 24

were scored for OA by a board-certified radiologist blinded as to treatment group. Radiographs

were scored as follows: 0 = a normal joint; 1 = a joint with radiographic evidence of instability

with no degenerative change; 2 = a joint with mild degenerative change and occasional

osteophytes; 3 = a joint with moderate degenerative change including osteophytes and

subchondral sclerosis; and 4 = a joint with severe degenerative change including osteophytes,

subchondral sclerosis, and bone remodeling (47).

Statistical Analysis

Statistical analysis was performed using a commercially available software package[k].

Age, weight and duration of lameness, data obtained from PSW analysis (stance time, stride

velocity, PVF, VI, and maximum peak pressure), and goniometric data (maximum flexion, maximum extension, and range of motion) were compared between subject groups using an independent group means test. Change in weight, PSW data and goniometric data was evaluated between time points within each subject group using a repeated measures ANOVA test for main effects and interactions. Data obtained from the BCS, lameness exam scores, VAS, CBPI (PS and PI), and radiographic scores were compared between subject groups at each time period by Non-Parametric Mann Whitney U' Test. Data obtained from the BCS, lameness exam scores, VAS, CBPI, and radiographic scores were evaluated between time points within each subject group by Friedman's test for nonparametric repeated measures.

The PVF score at baseline was used to stratify data and a mixed model was used to describe the change from baseline for the objective variables using analysis of covariance (48). An unequal variance model for the weeks measured and a first degree auto correlation was used to account for the correlation among the weeks within a dog. Dog within a group was used as a random effect. The baseline value and the number of cells were considered as covariates. Non-significant covariate variables were deleted from the model. ANCOVA was also used to compare the change in all variables at all post-injection time points from baseline between the treatment and placebo group. A commercially available software package[l] was used for all computations.

A post-hoc power analysis was performed for each variable using a freely available program[m] with $\alpha = 0.05$ and power=0.80. Data were reported as mean ± standard deviation. Significance was set at $p \leq 0.05$ in two tailed hypothesis testing. Effect sizes were reported as standardized effects using Cohen's d.

Results

Sixty eight client-owned dogs were evaluated for the study. Of these, 22 dogs (32.3%) were enrolled. Reasons for exclusion included a failure to meet the PSW criteria for PVF abnormalities (25 dogs), stifle pathology (16 dogs), hepatic disease (2 dogs), neurologic gait abnormalities (2 dogs), and upper respiratory disease (1 dog). Eleven male and eleven female dogs were enrolled. The average age of enrolled dogs was 8.0 years (range 1 year to 14 years). The average body weight was 35.0 kg (range 15.8 to 58.3 kg) and average body condition score was 3.5 (range 2 to 5). Three dogs were receiving NSAIDs during the study, seven dogs were receiving glucosamine and hyaluronic acid supplementation, six dogs were receiving opioids, and one dog was receiving acupuncture therapy. Based on the results of PSW analysis, 11 dogs were determined to have a lower PVF in the right leg and 10 had a lower PVF in the left leg. One dog had equal PVF in both hind limbs, with a combined 34% of body weight, and the right hind leg was selected at random to receive treatment. Nineteen dogs were visibly lame on initial assessment, and the remaining three had signs at home consistent with hip dysplasia (difficulty rising, 'bunny hopping' gait). No dogs had clinically significant abnormalities detected on complete blood count or serum biochemistry. Twenty one dogs had radiographic evidence of bilateral coxofemoral OA, with the remaining dog demonstrating unilateral OA.

Seventeen dogs completed the study. One dog from the placebo group was euthanized due to a diagnosis of splenic hemangiosarcoma approximately two weeks after SVF therapy, and data for this dog was censored from the study. Two dogs from the placebo group were withdrawn from the study after diagnosis of cranial cruciate ligament ruptures at the 8 week and 12 week evaluation, respectively. One dog in the placebo group did not present for follow-up evaluations beyond the 4 week time-point, and one dog from the treatment group was withdrawn from the study after the 12 week evaluation due to owner perceived worsening pain. These

animals had data included up to the time of study withdrawal. Two dogs had final follow-up evaluation performed at 32 weeks instead of 24 weeks.

Apart from the aforementioned cases, no dogs developed additional pathologies during the study. There were no acute SVF infusion-associated toxicities noted nor were any adverse reactions that could be attributed to SVF treatment. Two dogs (one in the placebo group and one in the treatment group) were noted to have increased lameness and pain on manipulation of the hip for up to one week after the treatment. In both cases, the pain resolved without additional treatment.

The mean live cell count injected as SVF as detected by cell viability counter was 341 million cells/mL (range 64 million to 584 million). MSC-like cells were isolated from all SVF isolates after 7-10 days in culture and no bacterial contamination was found. No significant differences were found in the viable cell count between placebo and treatment groups.

Average age between the treatment group (8.60 ± 4.13 years) and placebo group (7.46 ± 4.27 years) was not statistically different (p = 0.457). Average body weight at initial presentation between the treatment group (32.74 ± 9.13 kg) and placebo group (37.25 ± 12.39 kg) was not statistically different (p = 0.175). Mean duration of lameness prior to enrolling in the study between the treatment group (36.4 ± 31.7 months) and placebo group (49.9 ± 31.7) was statistically different (p=0.0351). There was no significant difference in body weight between groups at any time point during the study, although the treatment group tended to lose weight over time while the placebo group gained weight (Table 2.1).

Treatment and placebo groups were compared across all times for goniometric data (maximum flexion, maximum extension, and range of motion), VAS score (Table 2.1), BCS, lameness score, and CBPI scores; both PS and PI (Table 2.2); and radiographic scores (Table

2.3). The treatment group had a significantly lower BCS at 24 weeks compared to the placebo group (p = 0.036), but not at any other time point. The treatment group had a significantly lower PS at time 24 compared to the placebo group (p= 0.042) but not at any other time point. The change from baseline of the treatment group was significantly greater for both PS and PI at all time points compared to the placebo group, with a significant decrease in these variables found for the treatment group. The mean effect size for PS was 1.0-1.1. Similarly, the mean effect size for PI was 1.0-1.3.

When dogs were assessed as lame vs. non-lame a significant difference was found between treatment and placebo groups at all time point, with the placebo group having a higher percentage of lame dogs. No significant differences were found in, VAS score, goniometric data, or radiographic scores, or PI at any time points. No significant differences were found for VAS score, goniometric data, radiographic scores, or PI at any time points.

No significant differences were found between the treatment and placebo group at any time point for PVF, VI, stance time, stride velocity, or maximum peak pressure when either the treated or untreated (contralateral) limbs were assessed (Table 2.4). When the PVF data were stratified, a significant difference was seen in PVF at all time points between treatment and placebo groups for the animals in the lowest 25th percentile for PVF (Figure 2.1).

Effect size analysis was performed on the PSW data and shows that standardized effect size of dogs in the PVF 10th percentile was computed to be 0.38. The standardized effect size of dogs in the PVF 25th percentile was calculated to be 0.34.

When data were analyzed to look for effects within each group over time, no significant difference was seen in either group with respect to weight, BCS, lameness score, goniometric data, radiographic score, or PI. The VAS score for the treatment group was significantly greater

at 0 weeks than at 4, 8, or 24 weeks (p<0.05) but not at week 12. No significant differences were observed between VAS values in the placebo group. The PS score for the treatment group was significantly greater at 0 weeks than at 12 weeks (p = 0.011), but not when compared to any other time point. No significant differences were observed between PS values in the placebo group.

No significant differences were seen within groups at any time point with regard to PVF, VI, stance time, stride velocity, or maximum peak pressure of the treated limb. When evaluating the untreated limb, PVF was significantly higher at week 0 compared to week eight (p = 0.009) in the placebo group. VI was significantly higher at weeks 0, 12, and 24 when compared to week 8 (p = 0.026) in the placebo group. No significant differences were seen within the treatment group at any time point for any of the variables.

Post-hoc power analysis was performed on the PSW data and shows that the minimum number of dogs needed to detect a statistical difference between groups with an alpha of 0.05 and a power of 0.8 (PVF at 8 weeks) would be 82 dogs, assuming a 1:1 location of dogs between groups. The maximum number to detect a statistical difference (PVF at 4 weeks) would be 6884 dogs. Post-hoc power analysis performed on subjective criteria (VAS, CBPI, lameness score) shows that the minimum number of dogs needed to detect a statistical difference between groups (lameness score at 12 weeks) would be 30 dogs. The maximum number to detect a statistical difference (VAS at 24 weeks) would be 330 dogs. When designing a fully powered clinical study when PVF stratification data was used, and only the lowest 25th percentile is enrolled (e.g. dogs with a baseline PVF <38% of body weight), the minimum number needed to detect a significant difference (with a more stringent $\alpha = 0.025$ and power=0.90) is 25 dogs per group.

Table 0.1: Mean score and standard deviation of weight, VAS scores, and goniometric data at each time point

		0 Weeks	4 Weeks	8 Weeks	12 Weeks	24 Weeks
Weight (kg)	Treated	32.74 ± 9.13	32.5 ± 9.17	32.4 ± 9.26	32.17 ± 9.00	30.42 ± 9.26
	Placebo	37.25 ± 12.39	36.91 ± 12.01	39.1 ± 11.53	39.96 ± 12.52	40.43 ± 12.09
VAS Score (1 through 10)	Treated	3.35 ± 1.09	1.96 ± 0.98	1.91 ± 1.09	2.08 ± 1.35	1.70 ± 1.14
	Placebo	4.23 ± 2.24	2.43 ± 1.51	2.81 ± 1.53	2.60 ± 0.99	2.14 ± 1.60
Maximum flexion (degrees)	Treated	43.36 ± 7.36	42.27 ± 6.47	40.91 ± 8.31	43.64 ± 9.24	43.50 ± 8.83
	Placebo	42.46 ± 8.38	44.55 ± 10.60	42.22 ± 8.70	46.25 ± 8.76	45.71 ± 4.50
Maximum extension (degrees)	Treated	137.27 ± 16.64	145.00 ± 7.75	146.82 ± 11.89	148.64 ± 9.77	143.30 ± 13.70
	Placebo	136.09 ± 15.07	142.27 ± 22.73	143.89 ± 12.44	138.75 ± 15.53	140.00 ± 19.58
Range of motion (degrees)	Treated	93.91 ± 19.18	102.73 ± 6.84	105.91 ± 14.97	105.00 ± 16.43	99.80 ± 16.27
	Placebo	93.64 ± 17.84	97.73 ± 24.53	101.67 ± 16.20	92.50 ± 20.53	94.29 ± 21.49

There were no significant differences between treatment groups at $p \leq 0.05$.

Table 2.2: Median scores for BCS, lameness score and Canine Brief Pain Inventory scores at each time point

		0 Weeks	4 Weeks	8 Weeks	12 Weeks	24 Weeks
Body Condition Score (1 through 5)	Treated	4 (2.5 - 4.5)	4 (2.5 -5)	3 (2.5 - 4.5)	3.5 (3 -5)	**3 (2.5 - 4)***
	Placebo	3.5 (2 - 5)	3 (2 - 5)	4 (2.5 - 5)	4 (2.5 - 5)	**4.5 (3 - 5)***
Lameness Score (1 through 5)	Treated	3 (1 - 3)	3 (1 - 3)	1 (1 - 3)	1 (1 - 3)	1.5 (1 - 3)
	Placebo	3 (1 - 3)	3 (1 - 3)	3 (1 - 3)	3 (1 - 3)	3 (1 - 3)
CBPI Pain Severity	Treated	4.75 (0.25 - 7.25)	2.50 (0- 6.25)	1.75 (0 - 7)	1.00 (0 - 6.75)	**1.75 (0 - 7)***
	Placebo	5.00 (0 - 7.75)	4.38 (0 - 6.35)	3.50 (0 - 5.35)	5.50 (0 - 6.25)	**3.00 (0 - 8.75)***
CBPI Pain Interference	Treated	6.17 (0 - 8.67)	2.17 (0 - 75)	2.00 (0 - 8)	2.00 (0 - 8.83)	2.00 (0 - 8)
	Placebo	6.50 (0.17 - 9.67)	4.17 (0.17 - 95)	5.75 (0.17 - 8.17)	6.33 (0.5 - 8.33)	4.67 (0 - 9.83)

* Indicate significant differences between treatment groups. There were no other significant differences between treatment groups at $p \leq 0.05$.

Table 2.3: Median radiographic scores at weeks 0 and 24

		0 Weeks	24 Weeks
Radiographic Score(1-5)	Treated	2 (0-4)	3 (0-4)
	Placebo	2 (1-4)	4 (2-4)

There were no significant differences between treatment groups at p≤0.05.

Table 2.4: Mean ground forces data from pressure sensitive walkway at each time point

		0 Weeks	4 Weeks	8 Weeks	12 Weeks	24 Weeks
Peak Vertical Force (% body weight)	Treated	39.83 ± 6.11	38.66 ± 5.05	38.79 ± 5.51	40.17 ± 5.72	41.47 ± 4.38
	Placebo	41.18 ± 7.41	39.17 ± 9.32	34.26 ± 8.60	38.46 ± 6.45	40.86 ± 11.01
Vertical Impulse (% body weight x seconds)	Treated	14.21 ± 3.15	13.56 ± 2.52	13.63 ± 2.82	13.78 ± 3.00	13.47 ± 4.00
	Placebo	14.61 ± 2.78	12.98 ± 4.60	12.10 ± 4.89	14.76 ± 2.87	14.70 ± 3.39
Stance Time (seconds)	Treated	0.51 ± 0.11	0.49 ± 0.08	0.50 ± 0.09	0.49 ± 0.09	0.50 ± 0.08
	Placebo	0.52 ± 0.11	0.49 ± 0.12	0.53 ± 0.14	0.57 ± 0.12	0.56 ± 0.12
Stride Velocity (cm/second)	Treated	91.41 ± 16.15	98.51 ± 19.65	95.15 ± 18.47	96.59 ± 12.16	96.66 ± 13.07
	Placebo	94.13 ± 14.98	100.61 ± 13.41	93.74 ± 21.79	85.70 ± 20.19	91.44 ± 21.32
Maximum Peak Pressure (kg/cm²)	Treated	1.66 ± 0.54	1.54 ± 0.35	1.58 ± 0.36	1.59 ± 0.39	1.55 ± 0.42
	Placebo	1.71 ± 0.29	1.60 ± 0.41	1.48 ± 0.47	1.77 ± 0.24	1.71 ± 0.36

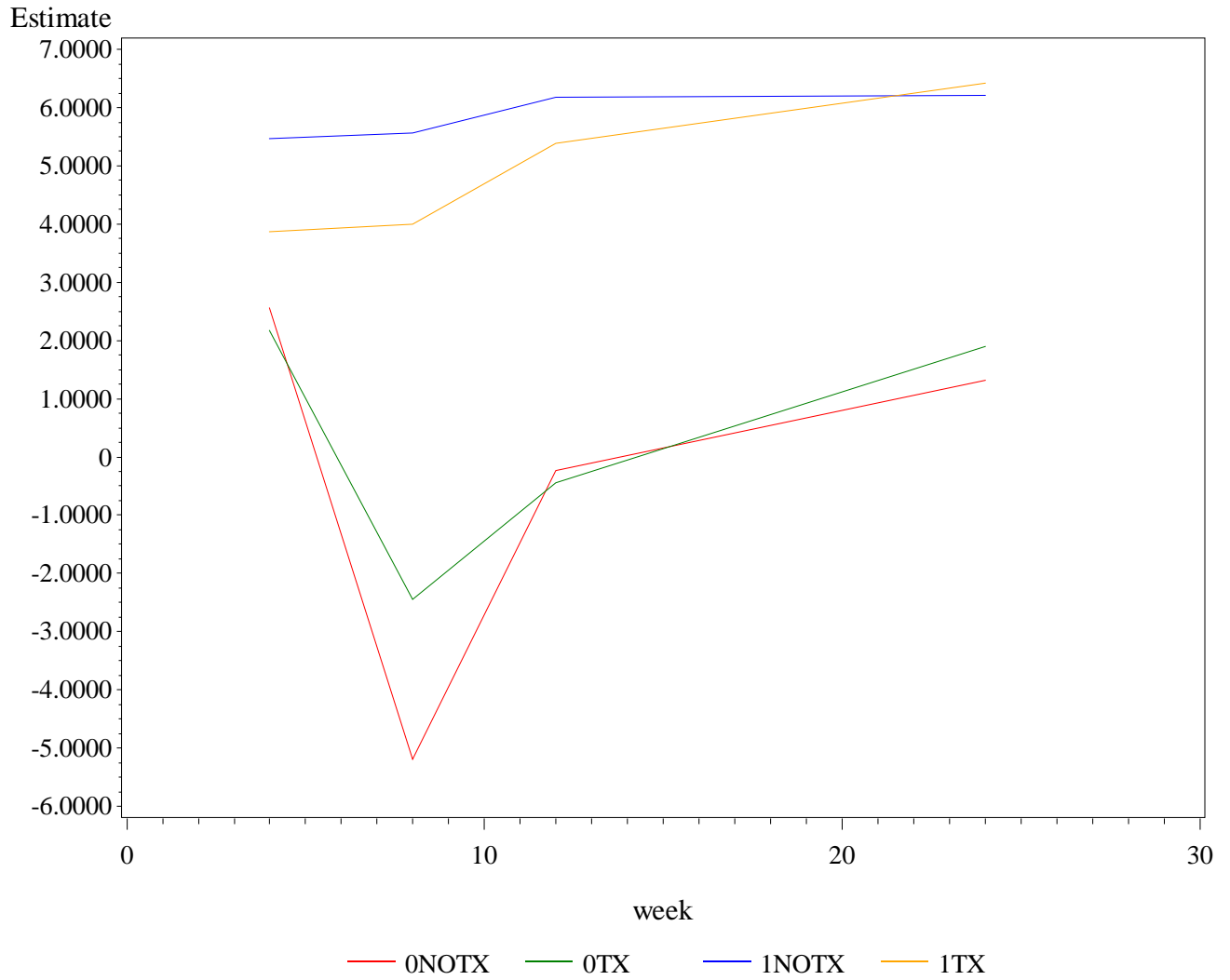There were no significant differences between treatment groups at p≤0.05.

Figure 0.1 Analysis of PVF Change from baseline in animals with lowest 25% of PVF – 0NOTX (untreated limb from placebo group), 0TX (treated limb from placebo group), 1NOTX (untreated limb from treatment group), 1TX (treated limb from treatment group)

Discussion

A number of publications exist that evaluate the use of MSC in the treatment of OA in laboratory or companion animals (25-33). To the authors' knowledge, only four veterinary studies have been conducted to assess the efficacy of SVF treatments for OA (39-42). While three of these four studies showed subjective improvements in joint range of motion and owner perceived functional outcome, none of the studies utilized kinetic data, such as that obtained via PSW analysis, as an outcome measure.

One aim of our study was to evaluate safety and efficacy of SVF/ PRP in coxofemoral OA. The safety of MSC treatment has been called into question, with concerns including neoplastic transformation of the cells, increased patient susceptibility to infection, embolism of the cells, and acute or chronic immune reactions to the cells themselves (34). One meta-analysis of human studies that utilized intravenous MSC therapy found that there was no increased risk of adverse effects with MSC therapy, with the exception of an increased risk of transient fever. (35) A more recent article found that after mice received an intravenous injection of 1.5×10^5 adipose derived MSCs there was an 85% mortality rate within 24 hours due to pulmonary thromboembolism (PTE). That report speculated that the MSCs expressed tissue factor which stimulated the extrinsic clotting cascade. Similar pro-coagulation effects were found in vitro when human adipose-derived MSCs were exposed human blood or plasma. (36) A few human cases of pulmonary thromboembolism associated with MSC therapy, one of them fatal, have been reported as well (37, 38). The safety of MSCs delivered via alternative routes and the safety of SVF have not yet been assessed, although previous veterinary studies do not report any adverse events (39-42). In the current study, the only adverse effect noted after joint injection was transient lameness and pain that resolved within one week. Because one dog each in the placebo and placebo groups experienced this complication, it is most likely an effect of joint

distension or inflammation caused by the arthrocentesis and injection of liquid into the joint, and not an effect of the SVF/ PRP. No specific diagnostic tests were pursued in dogs to rule out *de novo* neoplasia development, pulmonary embolism or other potential complications, but no dogs showed any unexpected clinical signs during the six month course of the study. One dog diagnosed with hemangiosarcoma after inclusion in this study was in the placebo group. Based on these 22 dogs, a combination of SVF and PRP appears to be safe for intra-articular and intravenous injection. Thus our safety observations are similar to those seen in previous canine studies of OA treatment with SVF.

Changes in body weight can significantly influence ground reaction forces in lame dogs. A study in which lame dogs were fed different diets to induce a weight change found that dogs that had an increase in body weight had a corresponding decrease in PVF values and dogs that had a decrease in body weight had a corresponding increase in PVF (49). For a 3.2 ± 1.5kg increase in weight, the change in PVF decreased $3.8 \pm 2.8\%$ body weight. In the current study, the dogs in the treatment group with the greatest improvement in PVF were also the dogs that lost the most weight. Whether the improvements in PVF are attributable solely to decreased body weight is unknown. The reason for the loss in body weight in the treatment group is also unknown. It is possible that weight loss is an as yet unknown effect of SVF/PRP therapy or that dogs in the treatment group had decreased pain and were more active. It is also possible that the observation that treatment dogs lost more weight is a type I error, especially since the body weight was never significantly different between groups.

Previous canine studies of OA treatment with SVF utilized subjective outcome measures exclusively, including veterinary lameness evaluation, pain on manipulation of the limb, subjective range of motion of the joint, and an owner completed functional disability score.

(38,39) In an effort to compare the results of the current study with the previous ones, several subjective outcome measures were evaluated at each time point, including a veterinary lameness score, a veterinarian completed VAS, a client completed CBPI, goniometric analysis and (at weeks 0 and 24), radiographic assessment of the pelvis.

Both the veterinary lameness scale (39) and VAS (46) have been previously validated for use in assessing lameness and pain in dogs. While these tests can be performed by owners, it has been found that the VAS lacks validity when performed by individuals untrained in recognizing clinical signs of pain (50). In an effort to avoid this complication, as well as to avoid interobserver variability, all VAS and lameness scores were conducted by one individual (DU). No significant differences in lameness scores were found between or within groups at any time point. The VAS for the treatment group was significantly greater at 0 weeks than at 4, 8, or 24 weeks, but not between any other time periods. This most likely represents an effect of the treatment on pain in the treated animals. VAS was performed after conducting a full physical examination and took into account lameness as well as signs of pain on manipulation of the limb. While the lameness severity may not have changed over time, it is possible that the subjective amount of pain on manipulation of the limb decreased in the treatment group, accounting for the significant difference in VAS score. If this is the case, the SVF/ PRP injection is associated with a rapid reduction in VAS evident by 4 weeks post-treatment which persisted for six months post-treatment. A similar trend was not found for the placebo group. The lack of a significant difference between the baseline and 12 week analysis may be due to a type II error.

In a previous study of SVF therapy for coxofemoral OA in dogs, a non-validated owner survey showed a greater percentage of improvement of treatment dogs compared to placebo controls (38). Mean effect sizes in that study were significant (greater than 0.8) for pain (1.57),

lameness at a trot (1.36), range of motion (1.45), and a composite score (1.34). In the current study, a CBPI was utilized. The CBPI has been validated as an owner assessment of pain associated with chronic osteoarthritis (43, 44). It is divided into a PS score which assesses the magnitude of pain an animal experiences, and a PI which assesses the degree to which pain effects daily activities. The treatment group had a significantly lower PS at week 24 compared to the placebo, and the PS score for the treatment group was significantly greater at 0 weeks than at 12 weeks. Change from baseline values was found to be significantly different at all post-injection time points for both PI and PS, with the treatment group having a greater decrease in these values. The mean effect size for PS was 1.0-1.1, and the mean effect size for PI was 1.0-1.3. These effect sizes, while significant, are lower than those of the aforementioned study, but this likely reflects the use of different outcome measures (a validated owner survey vs. veterinary lameness exam).

However, recent evaluation of the ability of the CBPI to detect significant improvement in osteoarthritic dogs treated with carprofen found that a decrease in PS of >1 and PI of > 2 resulted in the most statistical power to predict if a treatment would lead to response in an individual dog (44). In the present study, the mean PS score decreased >2.0 and the mean PI score decreased > 2.8 at all time points for the treatment group when compared to baseline. For the placebo group, the PS decreased >1 at eight weeks when compared to baseline, but at no other time points. Based on the results of that report, the changes observed may indicate a positive response to treatment.

Objective gait analysis using force plates or PSW have become an accepted technique in both human and veterinary medicine (51, 52) While these methods may hold a theoretical advantage over subjective measures, the ability to detect the true sensitivity or specificity of any

method to detect lameness is hampered by the lack of a gold standard test for comparison (53). Nevertheless, several studies have compared force plate or PSW data to subjective criteria (54-56). In a study of dogs with clinically induced right hind limb lameness, PVF and VI obtained from force plate analysis did not correlate significantly with either VAS or numerical rating scale lameness evaluations (54). While comparison between VAS and PVF approached significance, this was only true if the lamest dogs were included in the comparison. The authors concluded that the observers were not able to detect mild to moderate lameness as well as the force mat. Another study using a PSW found a correlation coefficient of only 0.14 between PVF and VAS for animals assessed 48 hours after cranial cruciate ligament surgery (55). While the lack of correlation does not indicate superiority of one method over another, the authors conjecture that a variable that is collected objectively and is generated by the patient should be superior to a variable that is collected subjectively and generated by an observer.

When all subjects in the treatment and placebo groups were compared, our study found no significant difference in any PSW variables of the treated limb between the treatment and placebo group or within either of these groups over time. When looking at the untreated (contralateral) limb of the placebo group, PVF was statistically greater at week 0 compared to week 8 and VI was statistically greater at weeks 0, 12, and 24 compared to week 8. Dogs in the study were predominantly affected by bilateral coxofemoral OA. Because OA can have waxing and waning periods of severity, it is possible that at the eight week time point, the untreated limb caused a clinically significant lameness worse than on the treated limb within the placebo group. However, this phenomenon should have been equally likely to affect the treatment group, where no statistical difference over time was seen. It is more likely the result of a type I error due to the low sample size. It was found that dogs in the placebo group tended to have a lower PVF at

week eight when compared to all other time points. While this finding was not significant, it was consistently observed when dogs were stratified based on baseline PVF. The reason for this observation is unclear. It would be expected that any pain due to saline injection would have resolved by eight weeks, and the four week time point had a higher PVF on average than the eight week time point. No specific event occurred between four and eight weeks to allow us to explain this observation.

When the data was stratified into quartiles based on PVF, it was found that the treated animals in the lowest 25th percentile (corresponding to a PVF at baseline examination less than 38& body weight) had significantly increased PVF at all time points compared to the placebo group. No significant differences were seen in the other quartiles. This may indicate that animals with the lowest PVF, and presumably the worst OA, may have a more clinically significant response to therapy with SVF/ PRP. It is possible, however, that this phenomenon represents regression towards the mean, where the most extreme variables tend to become closer to the mean with subsequent measurements. When a power analysis was performed, it was found that between 16 and 36 animals would be needed to detect significant differences between PVF, CBPI scores and VAS scores if we used dogs with < 38% PVF at baseline. Future studies may use this measure as a cutoff value to help increase their sensitivity.

Dogs enrolled in the study were of multiple breeds, ages, body weights, and body condition scores. Based on radiographic and clinical lameness evaluations, the dogs had varying degrees of severity of coxofemoral OA. A more homogenous population of dogs would be ideal to decrease variance of PSW analysis. Future studies will ideally select a cohort of dogs of similar age, body weight, breed and severity of OA.

As a pilot study, one goal of this project was to determine a sample size necessary to detect a statistically significant treatment effect on the analyzed variables. The enrollment of 22 dogs in the current study was not of sufficient power for us to be able to rule out a type I error for PSW variables, but was effective at estimating effect sizes and estimating the sample size needed for a fully powered trial. Using effect size observed in PVF < 38% at baseline, or CBI-PI or CBI-PS or lameness score, we estimate that we would need 25 dogs per group to see significant differences in PVF (p< 0.025, power of 0.9). The low number of patients enrolled also precludes us from analyzing the effect of sex, age, or severity of OA on response to treatment. It is possible that animals respond differently or not at all to SVF/ PRP therapy depending on these variables, but further studies with larger sample size are necessary in order to assess those variables.

Other limitations include the use of intravenous SVF and PRP injection in addition to intra-articular injection. MSCs may express receptors that allow them to target areas of tissue damage and inflammation (15). It is possible that the intravenous SVF cells migrated to both coxofemoral joints in bilaterally affected dogs and caused treatment effects in both joints, leading to a symmetric improvement in both joints which may be responsible for the lack of improvement noted on PSW analysis. However, if this were the case, it would be expected that both hindlimbs would have increased PVF and the forelimbs would have a decrease in PVF as weight shifted to the hind limbs from the fore limbs. This was not observed. A previous study evaluated the effect of PRP alone injected intra-articularly into osteoarthritic joints in dogs and found a significant increase in PVF at twelve weeks after injection compared to pretreatment values (23). Similar significant findings were not observed in this study without data stratification, although it is possible that significant differences observed in VAS and CBPI

values were due to PRP and not to SVF. The use of both SVF and PRP was chosen for our study because the commercially available protocol[h] utilizes both. Further studies with a larger sample population would be needed to assess whether SVF enhances or inhibits the effects of PRP.

One other limitation includes the concurrent use of NSAIDs and other disease modifying agents during the study in some dogs. It is possible that use of these treatments could have affected results. Ideally, dogs would have all been taken off analgesic medications and undergone a washout period prior to study enrollment. We elected to allow dogs to be continued on any previous medical management to avoid changes in their current lameness.

In summary, this study found that SVF with PRP did not cause any significant adverse reactions when injected intra-articularly and intravenously in dogs over a 24 week observation period. While some subjective improvements were noted in treatment animals, without data stratification of PVF data, this study failed to find significant improvements in PSW data in dogs treated with SVF/ PRP for coxofemoral OA. However, when the data were stratified by PVF at baseline, a treatment effect of SVF/ PRP correlated with the degree of OA was observed such that significant treatment effects were observed in dogs $< 38\%$ PVF at baseline. Additional studies with more patients should be conducted in order to be able to detect a statistically significant effect of the treatment on the analyzed variables.

Footnotes:

[a] Hi-Rez Versatek Walkway, Tekscan Inc, South Boston, MA 02127, USA.

[b] Baseline® Metal Goniometer - 180 Degree Range - 6 inch Legs - Robinson:

[c] Tekscan Pressure Measurement System Walkway Software 7.02, Copyright, Tekscan Inc., South Boston, MA, 02127, USA.

[d] hydromorphone- Westword, Eatontown, NJ 07724, USA

[e] acepromazine- Vedco, St. Joseph, MO 64507, USA

[f] PropoFlo - Abbott Laboratories, North Chicago, IL 60064, USA

[g] codeine sulfate - Roxane Laboratories, Inc., Columbus, OH 43216, USA

[h] Medivet Biologics LLC, Nicholasville, KY, 40356, USA

[i] Nexcelom Auto 2000 – Nexelcom Biosciences, Lawrence, MA 01843, USA

[j] Veterinary 0.9% Sodium Chloride Injection USP - Abbott Laboratories, North Chicago, IL 60064, USA

[k] WINKS SDA 6, Version 6.0.93, Texasoft Inc., Cedar Hill, TX 75104, USA

[l] Proc Mixed, SAS Institute, Inc., Cary, NC. USA

[m] G*Power - Version 3.1.9.2 – Franz Faul, Universitat Kiel, Germany

Figure Legends

Figure 2.1: Analysis of PVF Change from baseline in animals with lowest 25% of PVF –

0NOTX (untreated limb from placebo group), 0TX (treated limb from placebo group), 1NOTX

(untreated limb from treatment group), 1TX (treated limb from treatment group)

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Appendix A - Lameness Scoring based on Black et al*

Lameness

1 No lameness observed

2 Intermittent weight-bearing lameness

3 Persistent weight-bearing lameness

4 Persistent non–weight-bearing lameness

5 Ambulatory only with assistance

6 Nonambulatory

* Black LL, Gaynor J, Gahring D, et al. Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter controlled trial. Veterinary Therapeutics 2007; 8(4): 272-284.