Evaluation of Intravenously Delivered Allogeneic Mesenchymal Stem Cells for Treatment of Elbow Osteoarthritis in Dogs: A Pilot Study

Anastasia Olsen[1] Valerie Johnson[1,2] Tracy Webb[1,2] Kelly S. Santangelo[3] Steven Dow[1,2] Felix M. Duerr[1]

[1] Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado, United States
[2] Center for Immune and Regenerative Medicine, Department of Clinical Sciences, Colorado State University, Fort Collins, Colorado, United States
[3] Department of Microbiology, Immunology, and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado, United States

Address for correspondence Felix M. Duerr, DVM, MS, DACVS-SA, DACVSMR, Department of Clinical Sciences, James L. Voss Veterinary Teaching Hospital, Colorado State University, 300 W. Drake Rd, Fort Collins, CO 80523, United States (e-mail: Felix.Duerr@colostate.edu).

Vet Comp Orthop Traumatol 2019;32:173–181.

Abstract

Objectives The aim of this study was to evaluate the safety and collect pilot data measuring clinical effects of intravenously administered, adipose-derived, culture-expanded, allogeneic mesenchymal stem cells in dogs with elbow osteoarthritis.

Materials and Methods Dogs ($n = 13$) with naturally occurring elbow osteoarthritis received three intravenous doses of allogeneic canine mesenchymal stem cells via an open-label clinical trial. Primary outcome measures collected over a 6-month study period included objective gait analysis, accelerometry, owner questionnaires and joint fluid analysis.

Results No acute adverse events were observed following repeated intravenous treatment with allogeneic mesenchymal stem cells. A significant improvement in mean client-specific outcome measure (CSOM) activity score and CSOM behaviour score was observed when pre-treatment values were compared with post-treatment values (day >28). In contrast, mean peak vertical force significantly decreased from baseline to post-treatment (>day 28). Weekly activity counts did not show a significant difference between baseline to post-treatment time points. Synovial fluid biomarkers did not change during treatment, and labelled mesenchymal stem cells were rarely detected in synovial fluid samples collected after mesenchymal stem cell administration.

Clinical Significance For dogs with naturally occurring elbow osteoarthritis, intravenous administration of mesenchymal stem cells was clinically well tolerated. While some subjective outcome measures showed significant improvements, objective outcome measures did not confirm similar changes. Further research is needed before intravenous mesenchymal stem cells can be recommended as a treatment for elbow osteoarthritis in dogs.

Keywords
- cartilage degeneration
- cell-based therapies
- clinical lameness
- degenerative joint disease
- elbow osteoarthritis

received
June 16, 2018
accepted after revision
December 11, 2018

DOI https://doi.org/10.1055/s-0039-1678547.
ISSN 0932-0814.

Introduction

Osteoarthritis is one of the most common joint disorders in humans and dogs. Current treatment protocols may have suboptimal efficacy and undesirable side effects such as gastrointestinal toxicity, renal dysfunction and cardiovascular disease.[1] The limited ability of cartilage to repair may occur because endogenous stem cell populations become depleted or functionally altered during osteoarthritis, which results in a significant decrease in chondrogenic differentiation potential of these cells.[2] Abnormal endogenous stem cell function and increased susceptibility to degradative stimuli may occur in response to elevated levels of inflammatory cytokines in the joint.[3]

Non-steroidal anti-inflammatory drugs are often used in the management of osteoarthritis but do not necessarily improve joint function or slow disease progression.[4] Thus, there has been growing interest in the use of biological therapies such as mesenchymal stem cells for the management of osteoarthritis in dogs, horses and humans. Treatment with mesenchymal stem cells has been reported to improve osteoarthritis through a variety of mechanisms by reducing the production of pro-inflammatory cytokines,[5,6] stimulating endogenous stem cell populations[7] and producing factors to slow osteoarthritis progression in the short term.[8]

Administration of culture-expanded, autologous, adipose- or bone marrow-derived mesenchymal stem cells by intra-articular injection is the most commonly reported regenerative treatment protocol for canine osteoarthritis.[9–11] Anaesthesia or sedation is often required to perform intra-articular mesenchymal stem cell administration. For animals with multiple arthritic joints, intra-articular injections can be time-consuming and costly. Moreover, synovial fluid from arthritic joints is reported to be cytotoxic to cultured mesenchymal stem cells.[12]

Mesenchymal stem cells can exert systemic anti-inflammatory effects following intravenous administration in animal models and in humans with inflammatory disorders.[13] In addition, mesenchymal stem cells can migrate to sites of inflammation in response to chemokines such as stromal cell-derived factor 1 (SDF-1).[14] These anti-inflammatory effects are generated by both autologous and allogeneic mesenchymal stem cells.[7] Repeated delivery of fresh, culture-expanded, allogeneic mesenchymal stem cells is safe in other species.[15,16] Accordingly, an open-label clinical trial of intravenous allogeneic canine mesenchymal stem cells for the treatment of naturally occurring elbow osteoarthritis was conducted. This study was designed to evaluate safety and gather preliminary data regarding the efficacy of systemic mesenchymal stem cell administration as a novel approach to treat osteoarthritis in dogs.

Materials and Methods

Study Protocol

Animal protocols were approved by the Institutional Animal Care and Use Committee and the Clinical Review Board at Colorado State University. No incentive for participation in the study was provided; however, all cost associated with enrolment, administration of stem cells and data acquisition was covered by the study. Client-owned dogs were evaluated by a board-certified orthopaedic surgeon at the enrolment visit (day −28), and dogs with Grade 3 or greater lameness (Grade 0 = normal; Grade 1 = intermittent lameness when trotting, normal at walk; Grade 2 = consistent lameness when trotting, intermittent lameness noted at walk; Grade 3 = consistent lameness when walking; Grade 4 = toe touching lameness; Grade 5 = non-weight bearing lameness) attributable to elbow osteoarthritis were eligible for inclusion. Inclusion criteria were defined as body weight greater than 15 kg, consistent lameness for a minimum of four consecutive weeks immediately prior to enrolment, radiographic evidence of elbow osteoarthritis at the enrolment visit (based on interpretation and subjective grading by a board-certified radiologist as mild, moderate, or severe) and enrolment visit Canine Brief Pain Inventory (CBPI) values for pain severity score (PSS) and pain interference score (PIS) of greater than or equal to two.[17] If receiving osteoarthritis management at the time of enrolment, patients must have received a consistent osteoarthritis management protocol for 4 consecutive weeks immediately prior to enrolment, and owners had to agree to continue the same protocol throughout the study period. Exclusion criteria were defined as any intra-articular treatment or use of corticosteroids in the past 3 months, orthopaedic surgical treatment in the past 6 months, concurrent disease for which medications were currently prescribed that would interfere with osteoarthritis therapy, neurological disease (e.g. conscious proprioception deficits or visible ataxia affecting gait analysis), other orthopaedic disease causing pain or lameness and inflammatory arthropathies (i.e. autoimmune arthritis and infectious arthritis). Dogs with osteoarthritis in multiple joints were eligible for enrolment as long as the elbow joint was the most clinically affected. All dogs were required to wear a neck collar 24 hours a day and 7 days a week for attachment of an accelerometer. Lastly, the owners agreed to keep a daily activity and medication log and complete owner questionnaires throughout the study period. Dogs were required to discontinue non-steroidal anti-inflammatory drug therapy for 2 weeks prior to enrolment and could not receive non-steroidal anti-inflammatory drug therapy throughout the study period unless required for rescue/humane purposes. All study time points and outcome measures included are listed in ►**Fig. 1** and described below in detail.

Mesenchymal Stem Cell Culture, Characterization and Administration Protocol

Mesenchymal stem cells were derived from adipose tissue collected from the inguinal region of 10 anesthetized, purpose-bred research dogs used in a veterinary teaching laboratory. At the time of adipose tissue collection, the dogs underwent PCR and serological testing for infectious diseases: *Anaplasma phagocytophilum*, *Anaplasma platys*, *Borrelia burgdorferi*, *Dirofilaria immitis*, *Ehrlichia canis*, and *Ehrlichia ewingii* and *Hemoplasma spp*., *Ehrlichia spp*., *Bartonella spp*., and *Rickettsia spp*. In addition, a haematology and serum chemistry profile were obtained. Adipose tissue was utilized if donors were

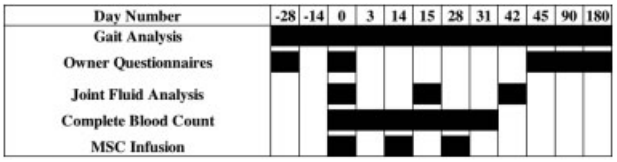

Fig. 1 Overview of data collection time points for all outcome measures.

negative for infectious disease and blood work was unremarkable. Adipose tissue was minced, cryogenically preserved until use, then thawed and collagenase-digested. The stromal vascular fraction was plated for expansion of mesenchymal stem cells as described previously.[18] Mesenchymal stem cells were harvested at passages three to five, washed, assessed for viability and administered within 30 minutes of harvesting. Mesenchymal stem cells were monitored for sterility via in house cultures for aerobic bacteria, fungi and mycoplasma. The cell surface phenotype of the mesenchymal stem cells was determined via flow cytometry (Gallios Flow Cytometer; Beckman Coulter, Inc., Brea, California, United States).[19] Tri-lineage differentiation of canine adipose derived mesenchymal stem cells was performed on three samples of adipose-derived mesenchymal stem cells processed as described above utilizing the StemPro Adipogenesis Differentiation Kit, the Chondrogenesis Differentiation Kit and the Osteogenesis Differentiation Kit (Life Technologies Corp.), according to manufacturer's instructions. Light microscopy was performed to confirm staining of osteogenic, adipogenic and chondrogenic cells (Olympus CKX41). Phenotyping of these samples to confirm the presence of common stem cell markers was performed as previously described[20] and the cells were confirmed to be positive for stem cell markers CD44, CD90 and CD105 and negative for haematopoietic stem cell markers CD45 and CD34. Subsequent cultures were considered to be mesenchymal stem cells based on commonly utilized properties of adherence to plastic and uniform fibroblastic phenotype.[20] Dogs received three intravenous infusions of mesenchymal stem cells, administered at 2-week intervals, via a peripheral vein catheter over 10 to 20 minutes. The dose of mesenchymal stem cells administered was 1 to 2 $\times$ 10^6 cells per kilogram body weight. Mesenchymal stem cells were resuspended in 10 to 20 mL Dulbecco's phosphate buffered saline (Dulbecco's Phosphate Buffered Saline; Sigma-Aldrich Co., St. Louis, Missouri, United States) (DPBS) containing 200 IU of heparin sulphate per 10 mL DPBS to prevent cell clumping. Animals were monitored closely for any complications during cell administration and for 20 minutes post-infusion, and owners were advised to supervise their dogs after discharge for adverse events.

Synovial Fluid Collection, Preparation and Cytokine Analysis

Synovial fluid was collected via a 22-gauge needle from the medial aspect of the affected elbow joint after sterile prep of the skin surface. Fluid samples underwent cytological evaluation by a board-certified clinical pathologist. Slides were stained with haematoxylin and eosin and graded according to degree of inflammation. Supernatants were collected from centrifuged synovial fluid and stored at –80°C prior to analysis. Previously validated commercial enzyme-linked immunosorbent assays specific for canine prostaglandin E2 (PGE 2) (Cayman Chemical) and matrix metalloproteinase-2 (MMP-2) (R&D) were utilized per manufacturers' instructions. Joint fluid cytokines were analysed if there were at least three time points of joint fluid available for analysis and the fluid was not grossly contaminated by blood. Joint fluid was frozen at –80°C within 30 minutes of collection and stored until all samples were collected. All samples were thawed once and all testing run on the same day.

Labelled Mesenchymal Stem Cell Tracking

For detection of mesenchymal stem cells migration into the joints, mesenchymal stem cells for the first or second infusion were labelled using a cell membrane dye. Mesenchymal stem cells were incubated with DiD (Vybrant DiD Cell Labeling Solution; Thermo Fisher Scientific, Inc., Waltham, Massachusetts, United States) for 15 minutes, washed three times in DPBS to remove unincorporated dye and then resuspended in DPBS and heparin prior to administration. Joint fluid was centrifuged at 5,000 $\times$ g and the supernatant removed and stored for biomarker analysis. The cell pellet was resuspended in 50 µL DPBS and 20 µL was spread onto a slide and allowed to air dry. The slide was then stained with nuclear stain DAPI (4′,6-diamidino-2-phenylindole), fixed in 1% paraformaldehyde (Affymetrix) and examined using an Olympus IX-83 confocal microscope. Joint fluid was examined prior to the first mesenchymal stem cell infusion, 24 hours after labelled mesenchymal stem cell infusion and again on day 42.

Owner Questionnaires

Validated questionnaires[21] including CBPI, Liverpool osteoarthritis in dogs and client-specific outcome measures (CSOM) were administered at day -28, 0, 45, 90 and 180. For CSOM activity score, owners listed up to five time- and place-specific activities that were problematic for their dogs due to osteoarthritis. The same list of activities was scored from 0 to 4 (0 = normal, 1 = A little problematic, 2 = quite problematic, 3 = severely problematic, 4 = impossible) at set time points. For CSOM behaviour score, owners listed up to three time- and place-specific behaviours that were problematic for their dogs due to osteoarthritis. The same list of behaviours was scored from 0 to 2 (2 = significantly more or less than normal, 1 = less or more than normal, 0 = normal). The same owner was required to complete the questionnaires at each time point to ensure continuity of patient assessment. All questionnaires were completed as an independent interview process prior to interaction with study personnel to prevent bias.

Gait Analysis

Gait analysis was performed using a validated[22] pressure mat (HRV Walkway 6 VersaTek System; Tekscan, Inc., South Boston, Massachusetts, United States) to measure peak vertical force (PVF) and vertical impulse (VI). Symmetry indices were calculated as previously described.[23] Dogs were walked over the walkway in a dedicated, isolated gait analysis area

(40 by 25 feet) and the first five valid trials were analysed. A valid trial was defined as a straightforward walk without stopping, hesitating, trotting or pacing, no head movement and maintenance of a constant speed. For all trials, velocity (0.8–1.3 m/s) and acceleration ($\pm$ 0.5 m/s^2) were representative of a walking gait.[24] Velocity was recorded at the enrolment visit, and all dogs were required to walk at the same velocity ($\pm$ 0.3 m/s) at each gait analysis recheck. Each gait trial was video recorded (EverFocus eZ.HD EQ 900 Camera; EverFocus Electronics Corp., Duarte, California, United States). Collection and analysis of gait data were performed by a single observer (AO).

Accelerometry

Activity monitoring was performed using a validated accelerometer (Animal Actical; Starr Life Sciences Corp., Oakmont, Pennsylvania, United States) mounted on a separate dog collar (One Inch Adjustable Dog Collar; Lupine, Inc., Center Conway, New Hampshire, United States) in a ventral location on the neck as previously reported.[25] Collar tightness was kept consistent for each dog throughout the study period. The accelerometer was attached by removing the metal ring on the collar used for leash attachment and securing the accelerometer with two zip ties (Eight Inch Cable Ties; Commercial Electric, Cleveland, Ohio, United States). Data collection began at day −28, and activity data were recorded continuously throughout the study period. The accelerometer epoch length was set to 30 seconds. All accelerometer data were recorded and analysed by a single observer (AO).

Statistical Analysis

All outcome measures were summarized in terms of means and standard deviations, stratified by assessment time points. A linear mixed effects model with animal-specific random effects was used to evaluate changes in all outcome measures between the baseline/pre-treatment assessment to the post-treatment assessments. All reported p-values are two-sided, and $p < 0.05$ was used to define significance. Statistical analyses were conducted using SAS software (SAS Institute Inc., Cary, North Carolina, United States), version 9.4.

Results

Animal Description

A total of 13 dogs (8 females, 5 males) were enrolled in the study. Five breeds were represented including the Labrador Retriever ($n = 5$), Bernese Mountain Dog ($n = 2$), Golden Retriever ($n = 2$), English Springer Spaniel ($n = 1$) and Newfoundland ($n = 1$). There were two mixed breed dogs, which included a Shar Pei/Labrador Retriever mix and a Collie/German Shepherd dog mix. All dogs were spayed or castrated. At the time of enrolment, the dogs had a mean body weight of 34.1 $\pm$ 11.6 kg (range, 18.6–60 kg) and were a mean age of 7.9 $\pm$ 3.6 years (range, 1.5–13 years).

All dogs were diagnosed with moderate ($n = 4$) or severe ($n = 9$) clinically relevant elbow osteoarthritis based on radiographical evaluation, physical examination and gait analysis on day –28. The affected joint was the right elbow for nine dogs and the left elbow for four dogs. At the initial enrolment visit, all dogs had signs of contralateral elbow osteoarthritis. In addition, eight dogs exhibited further signs of multi-joint osteoarthritis including the coxofemoral joint ($n = 5$), stifle ($n = 4$), tarsus ($n = 2$) and carpus ($n = 2$).

Nine of 13 dogs had been treated with a non-steroidal anti-inflammatory drug prior to enrolment. The non-steroidal anti-inflammatory drug previously used was carprofen (Rimadyl; Pfizer Animal Health, New York, New York, United States) ($n = 5$), meloxicam (Metacam; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri, United States) ($n = 3$) or deracoxib (Deramaxx; Elanco Animal Health, Greenfield, Indiana, United States) ($n = 1$). One dog had received intra-articular hyaluronic acid (Hyvisc; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri, United States) and triamcinolone (Vetalog; Boehringer Ingelheim Vetmedica Inc., St. Joseph, Missouri, United States) 2 years prior to enrolment. The same dog received intramuscular hyaluronic acid with chondroitin sulphate (Polyglycan; ArthroDynamic Technologies, Inc., Lexington, Kentucky, United States) 3 years prior to enrolment. Four dogs previously underwent elbow arthroscopy (one dog underwent elbow arthroscopy of the same joint on two separate occasions). All four dogs were diagnosed with a fragmented medial coronoid process, which was removed arthroscopically. At the time of enrolment, 12 dogs were receiving a consistent osteoarthritis management protocol as described in the inclusion criteria, and 1 dog was not receiving any therapy because the owner did not wish to start an osteoarthritis protocol. Osteoarthritis management therapies other than non-steroidal anti-inflammatory drugs included combinations of the following: gabapentin (Neurontin; Pfizer, Inc., New York, New York, United States) ($n = 7$), omega-3 fatty acids ($n = 7$), glucosamine hydrochloride with sodium chondroitin sulphate (Dasuquin;, Nutramax Laboratories Veterinary Sciences, Inc., Lancaster, South Carolina, United States and Cosequin; Nutramax Laboratories Veterinary Sciences, Inc., Lancaster, South Carolina, United States) ($n = 7$), polysulphated glycosaminoglycan (Adequan, Luitpold Pharmaceuticals, Inc., Shirley, New York, United States) ($n = 5$), tramadol hydrochloride (Tramadol hydrochloride; Amneal Pharmaceuticals LLC, Paterson, New Jersey, United States) ($n = 4$), multi-ingredient joint supplements (Glycoflex; VetriScience Laboratories, Essex Junction, Vermont, United States; Ligaplex; Standard Process, Inc., Palmyra, Wisconsin, United States) ($n = 2$), amantadine hydrochloride (Symmetrel; Alliance Pharmaceuticals, Chippenham, Wiltshire, United Kingdom) ($n = 1$) and a milk supplement (Duralactin; Veterinary Products Laboratories, Phoenix, Arizona, United States) ($n = 1$).

Complications

All dogs received all scheduled mesenchymal stem cell infusions. No adverse events were noted during or immediately after mesenchymal stem cell infusion prior to discharge in any of the treated animals. Four dogs did not have all data included for statistical analysis; two of these dogs were diagnosed with a cranial cruciate ligament tear on day 31 of the study and tibial plateau levelling osteotomies were

performed without complication. A third dog was diagnosed with a traumatic Grade III left lateral patellar luxation on day 128 of the study and underwent block recession trochleoplasty and lateral imbrication without complication. For the three dogs that underwent orthopaedic surgery for concurrent disease, data were evaluated until the diagnosis of concurrent orthopaedic disease. The fourth dog that did not complete the study began exhibiting signs of hyporexia and weight loss on day 38. Blood work (haematology and serum chemistry profile) at that time revealed an inflammatory leukogram. The dog returned to normal after several days. On day 61, the same dog developed left-sided Horner's syndrome and was subsequently diagnosed with a cranial mediastinal mass on metastatic thoracic radiographs. The owner elected euthanasia on day 76 due to decline in quality of life. Necropsy revealed a thymoma, a left renal cyst with mild interstitial lymphoplasmacytic nephritis, marked chronic hepatocellular hydropic degeneration and right ventricular dilation. Evaluation of the joints revealed diffuse severe osteoarthritis of the stifles, coxofemoral joints and elbows with periarticular and intra-articular osteophytosis, cartilage erosion and ulceration, synovial hyperplasia and capsular sclerosis. For this dog, data were included for analysis until day 45.

On day 41, one dog was presented to an emergency clinic for the evaluation of a suspected urinary tract infection due to signs of pollakiuria and haematuria. During evaluation, an incidental hepatic mass was found. Surgical biopsies of the hepatic mass and portal and splenic lymph nodes revealed T cell rich small B cell lymphoma, suspected to be indolent. The dog remained in remission until 455 days after completion of the study period when lethargy and epistaxis occurred. Immune-mediated thrombocytopenia was diagnosed, but due to lack of response to medical management and concern for quality of life, euthanasia and necropsy were performed. Necropsy revealed focal vasculopathy with extensive infarction of the tongue, hepatocellular vacuolar degeneration and extramedullary haematopoiesis.

Two dogs required rescue analgesia with non-steroidal anti-inflammatory drugs during the study period. One dog received a dose of meloxicam on day 17 and day 24 of the study. The other dog received a dose of meloxicam on day 29 and day 194 of the study. The remaining 11 dogs did not require rescue non-steroidal anti-inflammatory drugs throughout the study.

Outcome Measures

Gait data, accelerometer weekly total counts, pertinent blood work values and owner questionnaire scores are summarized in ►**Table 1** and ►**Figs. 2** and **3**. Individual data for each dog are included in Appendix Tables 1–16 (available in online version only). There was a significant decrease in mean CSOM activity score at 90 days ($p = 0.0172$), and a significant decrease in mean CSOM behaviour score at day 45 ($p = 0.0036$) and day 180 ($p = 0.0383$) when compared with baseline. A significant decrease in mean PVF of the affected limb was observed at 180 days ($p = 0.0256$). All other variables evaluated did not change significantly throughout the course of the study.

Four dogs had fluorescent mesenchymal stem cells identified in smears made of the cellular component of the joint fluid obtained 24 hours post-intravenous injection of labelled mesenchymal stem cells. Paired pre- and post-treatment synovial fluid samples without blood contamination were available for four dogs (►**Table 2**); cytology revealed no change in inflammation for one dog and variable changes in the other three dogs (decrease from moderate to mild [$n = 1$], decrease from moderated/marked to mild [$n = 1$] and decrease from mild to minimal [$n = 1$]). Comparison of joint fluid cytokine analysis from multiple study time points in four dogs revealed no significant differences in pre- and post-treatment concentrations of MMP2 ($n = 4$) or PGE2 ($n = 3$).

Table 1 Change from baseline/pre-treatment (day −28 to day 0) to post-treatment (day >28)

Outcome measure	Mean change	Standard deviation	*p*-Value
Peak vertical force (PVF)% body weight	−1.44	1.94	0.0256
Symmetry index for PVF	−0.93	2.82	0.2801
Vertical impulse (VI) body weight × second	−0.33	1.04	0.2991
Symmetry index for VI	−1.66	5.65	0.3317
Pain interference score	−0.35	1.09	0.294
Pain severity score	−0.41	0.82	0.1129
Liverpool osteoarthritis in dogs score	−0.07	5.04	0.9643
Client-specific outcome measure activity score	−0.21	0.21	0.005
Client-specific outcome measure behaviour score	−0.33	0.27	0.0014
White blood cell count (x10^3/μL)	0	1.38	0.9974
Lymphocyte count (×10^3/μL)	−0.15	0.39	0.1811
Monocyte count (×10^3/μL)	0.04	0.26	0.6326
Neutrophil count (×10^3/μL)	0.09	1.25	0.8051

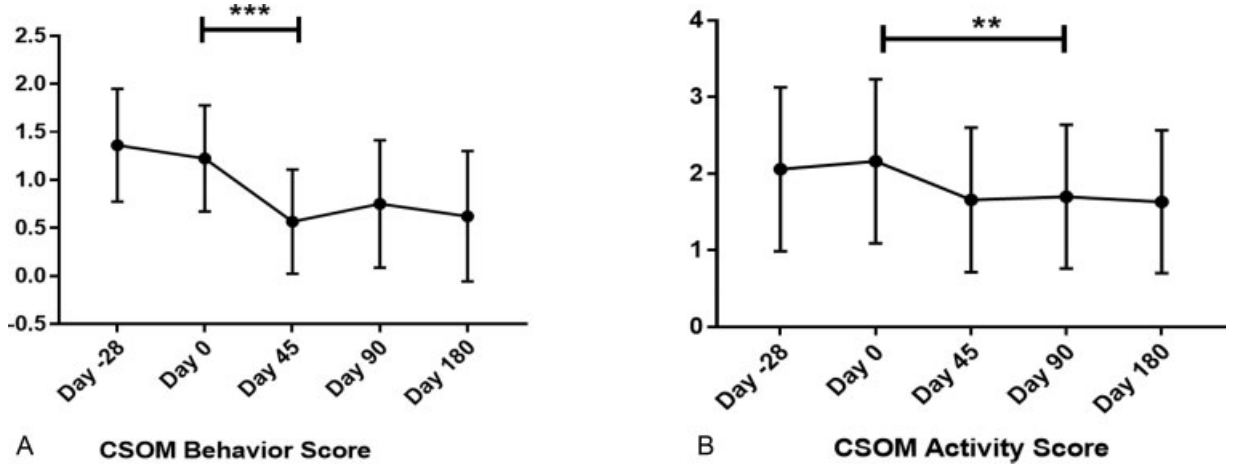

Fig. 2 Mean client-specific outcome measure (CSOM) Behaviour (**A**) and Activity (**B**) Scores from day −28 to day 180. Significant changes are indicated by $^{**}\leq 0.005$ and $^{***}\leq 0.0005$.

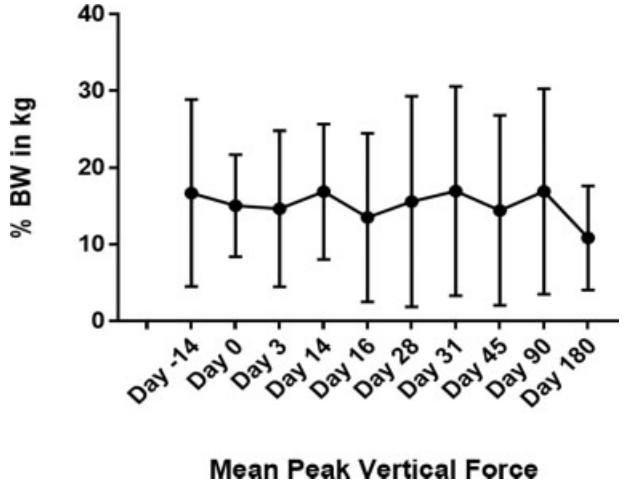

Fig. 3 Mean peak vertical force (PVF) from day −14 to day 180.

Discussion

For dogs with naturally occurring elbow osteoarthritis, the repeated use of intravenous allogeneic mesenchymal stem cells was easy to administer to awake dogs without adverse effects definitively attributable to the therapy. Overall there was little change in objective outcome measures assessed in this study. The CSOM owner questionnaire results and the successful discontinuation of non-steroidal anti-inflammatory drug therapy with minimal need for rescue administration may have been due to a real treatment effect, or attributed to the effects of owner bias in a non-blinded trial. A randomized, double-blinded, placebo-controlled, prospective study with a larger sample size is needed before any recommendations can be made for clinical application of intravenous allogeneic mesenchymal stem cells for elbow osteoarthritis in dogs.

While we found a significant improvement in CSOM activity and behaviour scores, there was no corresponding improvement in gait analysis parameters during the study. In fact, at day 180 PVF was significantly decreased. This may be a result of non-steroidal anti-inflammatory drug discontinuation or due to disease progression. Discontinuation of non-steroidal anti-inflammatory drug therapy was selected as an inclusion criteria to avoid confounding effects from concurrent therapy because non-steroidal anti-inflammatory drugs have been shown to alter gene expression in mesenchymal stem cells and may potentially affect the immunomodulatory effects.[26] Of the nine dogs dependent on non-steroidal anti-inflammatory drugs as part of their pre-study osteoarthritis protocol, two dogs required two doses each of non-steroidal anti-inflammatory drugs during the study period. It is noteworthy that dogs previously dependent on non-steroidal anti-inflammatory drugs were able to remain off these medications as the owners perceived that they had improved activity and behaviour based on CSOM scoring.

Gait analysis is currently considered the gold standard to evaluate improvement in dogs with osteoarthritis, but results can be conflicting. For example, PVF has been reported[27] to improve after non-steroidal anti-inflammatory drug use, while in other reports[4] it did not change. It has also been reported[28] that gait variation exists in normal dogs; thus, individual variation may have affected our interpretation of the gait data. The caregiver placebo effect[29] may also be responsible for the discrepancy between CSOM activity behaviour scores compared with gait analysis because owners were aware of the treatment. It is important to note that Liverpool osteoarthritis in dogs and CBPI did not show significant improvement. This is not surprising given previous sample size recommendations for clinical trials utilizing dogs with naturally occurring osteoarthritis.[4,17]

Mesenchymal stem cell administration has been widely evaluated for the treatment of ligament, tendon and joint injuries in humans and dogs.[30] However, in previous studies using mesenchymal stem cells to treat osteoarthritis, the cells were administered directly into affected joints. Systemic delivery of mesenchymal stem cells may exert an effect on osteoarthritis that is qualitatively different from that of intra-articular injection. When mesenchymal stem cells are administered intravenously, there is a greater interaction with the immune system as compared with intra-articular

Table 2 Cell counts in synovial fluid obtained from aspiration of elbow joints at various time points during treatment

Animal	Pre-treatment	Day 16	Day 42
Dog 1	Cell count: Mild ↑ (monocytes 86%, lymphocytes 13%, neutrophils 1%)	Cell count: Minimal ↑ (monocytes 68%, lymphocytes 30%, neutrophils 2%)	Not obtained
Dog 2	Cell count: Mild to moderate ↑ (monocytes 73%, lymphocytes 15%, neutrophils 12%)	Cell count: Mild to moderate ↑ (monocytes 83%, lymphocytes 17%)	Cell count: Mild to moderate ↑ (monocytes 87%, lymphocytes 11%, neutrophils 2%)
Dog 3	Cell count: Moderate ↑ (monocytes 65%, lymphocytes 30%, neutrophils 4%)	Cell count: Mild ↑ (monocytes 37%, lymphocytes 34%)	Cell count: Mild ↑ (monocytes 63%, lymphocytes 34%, neutrophils 3%)
Dog 4	Cell count: Moderate to marked ↑ (monocytes 67%, lymphocytes 32%, neutrophils 1%)	Cell count: Mild ↑ (monocytes 80%, lymphocytes 18%, neutrophils 2%)	Cell count: Mild ↑ (monocytes 75%, lymphocytes 22%, neutrophils 1%, eosinophils 2%)

Note: Cell count was estimated via manual counting of 10 fields of view and categorized as minimally (3,000–4,000 cells/μL), mildly (4,000–10,000 cells/μL), moderately (10,000–20,000 cells/μL), markedly (> 20,000 cells/μL), or severely (too numerous to count) increased.

use, which may lead to systemic anti-inflammatory effects.[18] Such systemic effects may reduce pain or inflammation at multiple sites, which is important as older dogs with osteoarthritis typically have multiple, concurrent musculoskeletal abnormalities. Mesenchymal stem cells are also capable of migrating to sites of inflammation following intravenous administration, as previous studies have demonstrated.[31–33] Chemokines such as SDF-1[34] and MCP-1[35] released by inflammatory stimuli provide a potent stimulus for mesenchymal stem cell recruitment, and these cytokines are known to be produced in the joints of animals with osteoarthritis.[36,37] Pulmonary trapping may interfere with this function,[38,39] which may explain why mesenchymal stem cells were detected in the joint fluid of only four dogs in our study. The time point selected for assessment (24 hours after intravenous mesenchymal stem cell injection) may also have played a role in this result. In a mouse model,[18] mesenchymal stem cells appeared to accumulate at sites of inflammation at later time points (5 days). Additionally, mesenchymal stem cells may track to the synovial membrane (which was not assessed in this study) and may not be released into the synovial fluid or the utilized labelling technique may not be adequate to detect all cells.

Analysis of cytokines and cell numbers in the synovial fluid revealed, at most, modest effects, and some effects could also be attributed to normal variation in cell counts in joints undergoing repeated arthrocentesis. Sample size for joint fluid analysis was severely limited due to difficulty in obtaining samples with adequate volume and without blood contamination. In the four dogs evaluated, three had a qualitative reduction in joint fluid cellularity following intravenous mesenchymal stem cells, but no change was noted in the concentrations of two biomarkers of osteoarthritis. Larger sample size and a control group are needed to determine the significance of these findings.

Two dogs in this study were diagnosed with neoplasia following administration of mesenchymal stem cells. Mesenchymal stem cell-induction of neoplasia or malignant transformation has been previously investigated, and previous reports[40,41] have not shown a correlation between mesenchymal stem cell therapy and development of neoplasia. Without an age-matched control group and a larger sample size, it is not possible to determine causation between mesenchymal stem cell administration and development of cancer in these two dogs. However, based on the observations in this study, future clinical research evaluating intravenous allogeneic mesenchymal stem cells should include further testing of dogs developing neoplasia to determine the cell of origin (donor cells versus host cells.)

Based on the success criteria for previously reported studies,[4,17] it is anticipated to have a change from baseline of at least 5% for PVF, a reduction of $\geq$1 for PSS and a reduction of $\geq$2 for PIS. Furthermore, based on the results of our pilot study, the expected standard deviation for the change is 12%, 2.0 and 2.5 for PVF, PSS and PIS respectively. Hence, the anticipated effect sizes for the primary endpoints range between 0.41 and 0.8. For PVF, PSS and PIS, a sample size of $n = 150$ (75 treated, 75 control), $n = 102$ (51 treated, 51 control) and $n = 42$ (21 treated, 21 control) is required to detect an anticipated effect size of at least 0.41 with 80% power at the two-sided 0.05 significance level respectively.

Conclusion

In conclusion, systemic, repeated administration of allogeneic mesenchymal stem cells in dogs with naturally occurring elbow osteoarthritis was clinically well tolerated and resulted in significant improvement in clinical CSOM scores, but no differences in objective outcome measures or other validated owner questionnaires. Results from this pilot study can be used to design additional randomized, double-blinded, placebo-controlled, prospective trials with an appropriate sample size to further evaluate the effects of intravenous mesenchymal stem cells as a treatment option for canine osteoarthritis.

Author Contribution

All authors contributed to conception of study, study design, acquisition of data, and data analysis and interpretation. All authors also drafted, revised and approved the submitted manuscript.

Conflict of Interest
Dr. Olsen reports grants from the Shipley Foundation and Eldred Foundation during the conduct of the study. Dr. Webb reports grants from the Shipley Foundation during the conduct of the study; personal fees and non-financial support from ReCellerate, Inc., outside the submitted work. Dr. Dow reports grants from Shipley Foundation, during the conduct of the study. Dr. Duerr reports grants from the Shipley Foundation and the Eldred Foundation during the conduct of the study. Dr. Johnson reports grants from Shipley Foundation, grants from Eldred Foundation, during the conduct of the study. Dr. Santangelo has nothing to disclose.

Acknowledgments
This study was supported by an award from the Shipley Foundation. We thank Jens Eickhoff for performing the statistical analysis.

References

1 Sinusas K. Osteoarthritis: diagnosis and treatment. Am Fam Physician 2012;85(01):49–56
2 Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum 2002;46(03):704–713
3 Fernandes JC, Martel-Pelletier J, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. Biorheology 2002;39 (1-2):237–246
4 Malek S, Sample SJ, Schwartz Z, et al. Effect of analgesic therapy on clinical outcome measures in a randomized controlled trial using client-owned dogs with hip osteoarthritis. BMC Vet Res 2012;8:185
5 Griffin MD, Ritter T, Mahon BP. Immunological aspects of allogeneic mesenchymal stem cell therapies. Hum Gene Ther 2010;21 (12):1641–1655
6 Frisbie DD, Kisiday JD, Kawcak CE, Werpy NM, McIlwraith CW. Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. J Orthop Res 2009;27(12):1675–1680
7 Freitag J, Bates D, Boyd R, et al. Mesenchymal stem cell therapy in the treatment of osteoarthritis: reparative pathways, safety and efficacy - a review. BMC Musculoskelet Disord 2016;17:230
8 Whitworth DJ, Banks TA. Stem cell therapies for treating osteoarthritis: prescient or premature? Vet J 2014;202(03):416–424
9 Black LL, Gaynor J, Adams C, et al. Effect of intraarticular injection of autologous adipose-derived mesenchymal stem and regenerative cells on clinical signs of chronic osteoarthritis of the elbow joint in dogs. Vet Ther 2008;9(03):192–200
10 Kiefer KM, Conzemius M, Pluhar GE. The development and use of canine adipose derived stromal and progenitor cells to treat osteoarthritis [dissertation]. St. Paul: University of Minnesota; 2013:93
11 Vilar JM, Batista M, Morales M, et al. Assessment of the effect of intraarticular injection of autologous adipose-derived mesenchymal stem cells in osteoarthritic dogs using a double blinded force platform analysis. BMC Vet Res 2014;10:143
12 Kiefer KM, O'Brien TD, Pluhar EG, Conzemius M. Canine adipose-derived stromal cell viability following exposure to synovial fluid from osteoarthritic joints. Vet Rec Open 2015;2(01):e000063
13 Zhang J, Huang X, Wang H, et al. The challenges and promises of allogeneic mesenchymal stem cells for use as a cell-based therapy. Stem Cell Res Ther 2015;6:234
14 Li F, Niyibizi C. Engraftability of murine bone marrow-derived multipotent mesenchymal stem cell subpopulations in the tissues of developing mice following systemic transplantation. Cells Tissues Organs 2016;201(01):14–25
15 Quimby JM, Webb TL, Randall E, Marolf A, Valdes-Martinez A, Dow SW. Assessment of intravenous adipose-derived allogeneic mesenchymal stem cells for the treatment of feline chronic kidney disease: a randomized, placebo-controlled clinical trial in eight cats. J Feline Med Surg 2016;18(02):165–171
16 Quimby JM, Webb TL, Habenicht LM, Dow SW. Safety and efficacy of intravenous infusion of allogeneic cryopreserved mesenchymal stem cells for treatment of chronic kidney disease in cats: results of three sequential pilot studies. Stem Cell Res Ther 2013;4(02):48
17 Brown DC, Bell M, Rhodes L. Power of treatment success definitions when the Canine Brief Pain Inventory is used to evaluate carprofen treatment for the control of pain and inflammation in dogs with osteoarthritis. Am J Vet Res 2013;74(12):1467–1473
18 Johnson V, Webb T, Norman A, et al. Activated mesenchymal stem cells interact with antibiotics and host innate immune responses to control chronic bacterial infections. Sci Rep 2017;7(01):9575
19 L Ramos T, Sánchez-Abarca LI, Muntión S, et al. MSC surface markers (CD44, CD73, and CD90) can identify human MSC-derived extracellular vesicles by conventional flow cytometry. Cell Commun Signal 2016;14:2
20 Chow L, Johnson V, Regan D, et al. Safety and immune regulatory properties of canine induced pluripotent stem cell-derived mesenchymal stem cells. Stem Cell Res (Amst) 2017;25:221–232
21 Belshaw Z, Asher L, Dean RS. Systematic review of outcome measures reported in clinical canine osteoarthritis research. Vet Surg 2016;45(04):480–487
22 Besancon MF, Conzemius MG, Derrick TR, et al. Comparison of vertical forces in normal greyhounds between force platform and pressure walkway measurement systems. Vet Comp Orthop Traumatol 2003;16:153–157
23 Kim J, Kazmierczak KA, Breur GJ. Comparison of temporospatial and kinetic variables of walking in small and large dogs on a pressure-sensing walkway. Am J Vet Res 2011;72(09):1171–1177
24 Horstman CL, Conzemius MG, Evans R, Gordon WJ. Assessing the efficacy of perioperative oral carprofen after cranial cruciate surgery using noninvasive, objective pressure platform gait analysis. Vet Surg 2004;33(03):286–292
25 Hansen BD, Lascelles BD, Keene BW, Adams AK, Thomson AE. Evaluation of an accelerometer for at-home monitoring of spontaneous activity in dogs. Am J Vet Res 2007;68(05):468–475
26 Almaawi A, Wang HT, Ciobanu O, et al. Effect of acetaminophen and nonsteroidal anti-inflammatory drugs on gene expression of mesenchymal stem cells. Tissue Eng Part A 2013;19(7-8):1039–1046
27 Moreau M, Dupuis J, Bonneau NH, Desnoyers M. Clinical evaluation of a nutraceutical, carprofen and meloxicam for the treatment of dogs with osteoarthritis. Vet Rec 2003;152(11):323–329
28 Hans EC, Zwarthoed B, Seliski J, Nemke B, Muir P. Variance associated with subject velocity and trial repetition during force platform gait analysis in a heterogeneous population of clinically normal dogs. Vet J 2014;202(03):498–502
29 Conzemius MG, Evans RB. Caregiver placebo effect for dogs with lameness from osteoarthritis. J Am Vet Med Assoc 2012;241(10): 1314–1319
30 Fortier LA. Stem cells: classifications, controversies, and clinical applications. Vet Surg 2005;34(05):415–423
31 Nagaya N, Fujii T, Iwase T, et al. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 2004;287(06):H2670–H2676
32 Kim Y, Jo SH, Kim WH, Kweon OK. Antioxidant and anti-inflammatory effects of intravenously injected adipose derived mesenchymal stem cells in dogs with acute spinal cord injury. Stem Cell Res Ther 2015;6:229
33 Petchdee S, Sompeewong S. Intravenous administration of puppy deciduous teeth stem cells in degenerative valve disease. Vet World 2016;9(12):1429–1434

34 Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003;362(9385):697–703
35 Wang L, Li Y, Chen X, et al. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. Hematology 2002;7(02):113–117
36 Fernandez-Pernas P, Rodríguez-Lesende I, de la Fuente A, et al. CD105+-mesenchymal stem cells migrate into osteoarthritis joint: An animal model. PLoS One 2017;12(11):e0188072
37 Favero M, El-Hadi H, Belluzzi E, et al. Infrapatellar fat pad features in osteoarthritis: a histopathological and molecular study. Rheumatology (Oxford) 2017;56(10):1784–1793
38 Spriet M, Hunt GB, Walker NJ, Borjesson DL. Scintigraphic tracking of mesenchymal stem cells after portal, systemic intravenous and splenic administration in healthy beagle dogs. Vet Radiol Ultrasound 2015;56(03):327–334
39 Fischer UM, Harting MT, Jimenez F, et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. Stem Cells Dev 2009;18(05):683–692
40 Centeno CJ, Al-Sayegh H, Freeman MD, Smith J, Murrell WD, Bubnov R. A multi-center analysis of adverse events among two thousand, three hundred and seventy two adult patients undergoing adult autologous stem cell therapy for orthopaedic conditions. Int Orthop 2016;40(08):1755–1765
41 Aanstoos ME, Regan DP, Rose RJ, Chubb LS, Ehrhart NP. Do mesenchymal stromal cells influence microscopic residual or metastatic osteosarcoma in a murine model? Clin Orthop Relat Res 2016;474(03):707–715