

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\*

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## CLINICAL RELEVANCE

Autologous stem cell therapy in the field of regenerative veterinary medicine involves harvesting tissue, such as fat, from the patient, isolating the stem and regenerative cells, and administering the cells back to the patient. Autologous adipose-derived stem cell therapy has been commercially available since 2003, and the current study evaluated such therapy in dogs with chronic osteoarthritis of the hip. Dogs treated with adipose-derived stem cell therapy had significantly improved scores for lameness and the compiled scores for lameness, pain, and range of motion compared with control dogs. This is the first randomized, blinded, placebo-controlled clinical trial reporting on the effectiveness of stem cell therapy in dogs.

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## INTRODUCTION

Advances in understanding of the biology of adult stem cells have attracted the attention of the biomedical research community, including those studying osteoarthritis (OA).<sup>1</sup> Autologous adult stem cells are immunologically compatible, can be harvested from a variety of sources, including bone marrow and adipose tissue,<sup>1</sup> and have no ethical issues related to their use. Mesenchymal stem cells (MSCs) derived from bone marrow (BM-MSCs) and adipose tissue (AD-MSCs) are the most highly characterized and are considered comparable.<sup>2</sup> Both have demonstrated broad multipotency with differentiation into a number of cell lineages, including adipo-, osteo-, and chondrocytic lineages.<sup>2</sup> However, the easy and repeatable access to subcutaneous adipose tissue, the simple isolation procedure, and the approximately 500-fold greater numbers of fresh MSCs derived from equivalent amounts of fat versus bone marrow provide a clear advantage in using AD-MSCs over BM-MSCs.<sup>3,4</sup> Isolated AD-MSCs can also be easily cryopreserved.<sup>3</sup>

The area of AD-MSC use for regenerative medicine has been the focus of many recent reviews, underlining the rapid pace of this field.<sup>2-8</sup> Isolation of cells from adipose tissue entails mincing and washing, followed by collagenase digestion and centrifugation.<sup>8,9</sup> The pellet formed from centrifugation is deemed the stromal vascular fraction (SVF), which is resuspended and used as the treatment modality. The SVF contains a heterogeneous mixture of cells including fibroblasts, pericytes, endothelial cells, circulating blood cells, and AD-MSCs.<sup>8,10-12</sup> As a result of the cells' "minimally manipulated" nature, many autologous stem cell therapies do not require an FDA drug approval application.

Veterinarians have used autologous AD-MSCs to treat tendon and ligament injuries and joint disease in horses on a commercial basis since 2003.<sup>13-15</sup> Studies and anecdotal clinical experience with more than 2,500 horses demonstrate that autologous AD-MSC therapy helps horses with tendon and ligament injuries.<sup>13-16</sup> In a blinded, placebo-controlled study, Dahlgren and Nixon and colleagues demonstrated statistically significant improvement in inflammatory cell infiltrate, collagen fiber uniformity, polarized collagen fiber crimping, overall tendon healing score, and collagen oligomeric matrix protein scores in an equine tendonitis model.<sup>15,16</sup> Used commercially on more than 2,500 horses with no significant systemic adverse events reported and less than 0.5% local tissue reactions (as of December 12, 2006), autologous AD-MSC therapy has been shown to be reasonably safe and therapeutically successful.

A number of recent publications provide evidence of therapeutic success with stem cell therapy in tendon or ligament injuries and degenerative joint disease in other species.<sup>1,17-19</sup> Nathan and colleagues demonstrated that AD-MSCs in a fibrin carrier were able to fill osteochondral defects created in rabbit femoral condyles better than fibrin carrier alone, and the biomechanical performance of the AD-MSC-treated group was clearly superior as well.<sup>19</sup> In a model of OA in the goat, BM-MSC therapy resulted in regeneration of the meniscal tissue and retardation of the normal progression of OA seen in the model.<sup>17</sup> Cell-treated joints had

marked meniscal regeneration with implantation of the BM-MSCs and a reduction in degeneration of the articular cartilage, osteophyte remodeling, and subchondral sclerosis. Based on scientific evidence and the therapeutic success in horses, veterinarians are now beginning to use regenerative medicine to treat similar conditions in dogs, including OA.

OA is the most common cause of chronic pain in dogs, with more than 20%, or 10 to 12 million dogs, afflicted in the United States at any time.<sup>20-22</sup> OA is characterized by degeneration of the articular cartilage, with loss of matrix, fibrillation, and formation of fissures, and can result in complete loss of the cartilage surface.<sup>23</sup> Chondrocytes, the only cells of articular cartilage, maintain homeostatic synthesis and degradation of the extracellular matrix via the secretion of macromolecular components (collagen, glycosaminoglycans, and hyaluronic acid) and modulation of the extracellular matrix turnover. Chondrocyte secretion and release of lytic and tissue-damaging mediators (cytokines, free radicals, proteases, prostaglandins) are controlled by a balance of anabolic and reparative substances (growth factors, inhibitors of catabolic cytokines) and inhibitors of degradative enzymes.<sup>23</sup> In OA, there exists an overproduction of destructive and proinflammatory mediators relative to the inhibitors, resulting in a balance in favor of catabolism rather than anabolism, which in turn leads to the progressive destruction of articular cartilage.<sup>23</sup>

Scientific studies and clinical experience with OA therapy in dogs suggest that NSAIDs, the current cornerstone of care, often do not provide complete pain relief.<sup>24-28</sup> In contrast to drug therapy, cellular therapies such as AD-MSC therapy do not rely on a single target receptor or pathway for their action. Cellular therapy functions trophically by secreting cytokines and growth factors<sup>29</sup> and by recruiting endogenous cells to the injured site, and it may promote cellular differentiation into the resident lineages.<sup>8</sup> MSCs “communicate” with the cells of their local environment, can suppress immunoreactions, and inhibit apoptosis, and new data now demonstrate that BM-MSCs can deliver new mitochondria to damaged cells, thereby rescuing aerobic metabolism.<sup>8,30</sup> Taken together, AD-MSCs respond to the local microenvironment in a manner that in many cases is demonstrated to enhance healing. The purpose of this blinded, randomized, placebo-controlled, multicenter study was to evaluate the clinical effect of a single intraarticular injection of adipose-derived stem and regenerative cells in dogs with lameness associated with OA of the coxofemoral joints.

## **MATERIALS AND METHODS**

### **Study Population**

Four companion animal regional referral veterinary practices in the San Diego area, Chicago, and Colorado Springs participated in this randomized, double-blinded, placebo-controlled trial that included outpatient dogs with OA of the coxofemoral joint. Twenty-one dogs (14 females and 7 males) ranging in age from 1 to 11 years were recruited based on the presence of bilateral coxofemoral joint OA with a minimum duration of 6 months. The breeds included Akita, boxer, German shepherd mix, Gordon setter, Great Pyrenees, Labrador retriever, rottweiler, schnauzer mix, standard poodle, Aussie mix, collie mix, golden retriever, puli, and Weimaraner; body weights ranged from 25 to 110 lb.

Before enrollment, all dogs underwent routine clinical chemistry and hematology evaluation to ensure overall health. Study animals demonstrated gait changes characteristic of OA, including persistent lameness at a walk and trot, pain on passive manipulation of the affected joint(s), and limited range of motion with pain at less than full range of passive motion. Finally, dogs demonstrated functional disabilities, including level of stiffness as measured by willingness to walk and run.

Each qualified case demonstrated pretreatment radiographic evidence of degenerative joint disease of grade 2 or higher on the following radiographic scoring scale:

- 0** = Normal joint
- 1** = Radiographic evidence of instability; no degenerative change
- 2** = Mild degenerative change (occasional osteophytes)
- 3** = Moderate degenerative change (osteophytes, subchondral sclerosis)
- 4** = Severe degenerative change (osteophytes, subchondral sclerosis, bone remodeling)

Dogs were excluded from the study if they had a history of coxofemoral joint surgery; very severe hip dysplasia with functional luxation; a history of spontaneous luxation or a likelihood of spontaneous luxation during the 6 months of the study; concurrent disease, such as a fungal, bacterial, or viral infection; malignant neoplasia; or any severe systemic disease that would confound interpretation of treatment effects.

Dogs on concomitant therapy, such as NSAIDs, were required to be on these medications for at least 14 days before enrollment in the study and to remain on the drugs at the same level throughout the study. Hyaluronic acid and polysulfated glycosaminoglycan injections and such alternative treatments as chiropractic and acupuncture, if used, were discontinued in all dogs in both groups for 10 days before enrollment in the study and were not administered during either phase of the study period. Two dogs were disqualified during the study because of inadvertent administration or removal of NSAIDs, which would preclude evaluation.

To be eligible, the dogs had to be cared for by attentive owners who agreed by informed consent to participate in this clinical study, to follow a set schedule of veterinary appointments, and to observe their dog for the entire study period.

### **Treatments**

The in-house laboratory at Vet-Stem prepared the test treatment material for each study dog. Lab technicians isolated autologous AD-MSCs and regenerative cells from a minimum of 23 g of fat collected from each dog by the investigator. Adipose was collected from both treatment and control dogs to maintain blinding. Laboratory personnel provided the test and control material to the investigator in two covered, sterile 1-ml syringes. Each dog received either 0.6 ml of phosphate buffered saline (PBS; control dogs) or a suspension of 4.2 million (MM) to 5 MM (depending on cell yield) viable cells prepared from the dog's own fat tissue in 0.6 ml PBS/joint. The veterinarians injected the hip joints at the midpoint of the proximal edge of the greater trochanter of the femur. One dog received 4.2 MM viable cells/joint; all other dogs received 5 MM cells/joint. The adipose samples from the control animals were also processed, and the viable nucleated cells were cryopreserved for use later. Laboratory technicians also prepared and archived a sample of the cell preparation from each case for additional study and prepared two saline syringes to flush the test or control article through the needle. The Vet-Stem clinical document coordinator prepared randomization sheets that were stratified by investigational center to ensure balance between treated (Group A) and control (Group B) dogs within centers. Dogs were assigned to a group during the receiving process for the sample according to the randomization sheet for the investigator. The Vet-Stem clinical document coordinator maintained the administration code throughout the study until the day 90 examination was concluded or in the event an animal was withdrawn from the study.

Dogs in Group A received a single intraarticular injection of the fresh test treatment material in each hip joint on day 0, and dogs in Group B received a single intraarticular injection of the placebo material in each hip on day 0. Neither the owners nor the investigators had knowledge of group assignments.

Owners were counseled to leash-walk their dogs twice daily. However, one dog in the test group was allowed to run and one had free run of a large pen. One control dog was leash-walked and allowed to swim.

### **Stem and Regenerative Cell Preparation**

#### ***Adipose Tissue Collection***

Adipose tissue was collected from the abdominal, inguinal, or thoracic wall regions of the dog. A small surgical incision (5 cm) was made aseptically after the patient was anesthetized. The adipose tissue was resected by scalpel or surgical scissors and placed into a labeled sterile tube containing 15 ml of PBS. The sample tube was placed in a validated, temperature-controlled 2°C transport box specially fitted with a frozen cold pack and shipped by overnight express courier to the Vet-Stem laboratory for processing.

#### ***Tissue Processing and Stem and Regenerative Cell Isolation***

Adipose tissue was washed with PBS, then minced and washed several times with PBS to remove debris and excess blood. The minced tissue was mixed well. Enzymatic digestion was performed by use of a combination of collagenase and hyaluronidase at 37°C for 50 minutes with agitation. The mixture was

centrifuged at 400  $\times$ g for 15 minutes, and the cell pellet was resuspended in PBS a total of four times. An aliquot of the final cell suspension was assessed for viability (trypan blue exclusion method) and total nucleated cell yield. This constitutes the SVF preparation. Vet-Stem internal data demonstrate that the mean CFU–fibroblast (CFU-F) percentage for canine regenerative cells is 1.72%, which is within the reported range of 1% to 4% CFU-F<sup>4</sup> for human AD-MSCs and far greater than the 0.001% to 0.01% CFU-F reported for human bone marrow.<sup>12</sup> Recent phenotypic cluster of differentiation (CD) marker analysis data reported from three independent laboratories demonstrate an approximate mean of 30% AD-MSC (CD34<sup>+</sup>, CD31<sup>-</sup>, CD146<sup>-</sup>) in human SVF and approximately 1% to 10% other “progenitor” cell types, including a pericyte cell fraction.<sup>10–12</sup> Canine fresh SVF contains approximately 85.6% mononuclear cells that do not fall into the hematopoietic lineage. Further characterization of canine SVF will be completed as canine CD marker reagents become available.

### **Evaluations**

Veterinary evaluation incorporated history, physical examination, and lameness examination including joint mobility and notation of pain on manipulation, a modified version of published criteria.<sup>27</sup> Clinical outcome measures were based on veterinary orthopedic examination evaluation using a numerical rating scale based on a standardized questionnaire (Figure 1). Baseline results for both owner and veterinary evaluations were recorded between 2 and 14 days before the dogs received either the test or control preparation by intraarticular injection. Follow-up visits to the veterinary clinic were required at 30, 60, and 90 days after the dog's intraarticular injection. At each visit, owners were also asked to complete a numeric rating scale (1 [best] to 5 [worst]) as part of a standard questionnaire adapted from the Cincinnati Orthopedic Disability Index,<sup>31</sup> which included evaluation of the following 13 parameters: walk, run, jump, turning suddenly, getting up from lying down, lying down from standing, climbing stairs, descending stairs, squatting to urinate or defecate, stiffness in the morning, stiffness in the evening, difficulty walking on slippery floors, and willingness to play voluntarily.

### **Statistical Evaluation**

The statistical significance of changes in scores over time for each parameter in each treatment group was tested by one-way repeated measures analysis of variance (ANOVA). In the rare instances in which the data were not normally distributed, results were substantiated by the nonparametric Friedman repeated measures ANOVA on rank sum test. Comparisons of responses between treatment and placebo groups were made by two-way repeated measures ANOVA with treatment and time as grouping variables. Post hoc comparisons were made by the Tukey test. The data were analyzed using commercially available statistical software (SigmaStat 3.5, Systat Software, Point Richmond, CA) at the nominal .05 level of significance.

To determine the magnitude of the response, the “effect size,” defined as the difference between the treatment and placebo response divided by the standard deviation of the placebo response,<sup>32</sup> was calculated for each parameter at each posttreatment evaluation time. Originally developed for the behavioral sciences,<sup>33</sup> effect size is a unitless measure of the degree to which the apparent treatment effect exceeds the placebo effect, wherein an effect size of 0.2 or less is considered a “small” effect and 0.8 or more is a “large” effect.

## **RESULTS**

Eighteen dogs completed the 90-day study. Two dogs were removed from the study as a result of either receiving or discontinuing an NSAID, and one dog was lost from the study because of other compounding medical issues. Both test article and control article were well tolerated by the dogs, with the exception of two placebo-treated dogs, both from the same investigator, that demonstrated biting and scratching at the injection sites of short duration. Each case resolved within 48 hours, and the cause was postulated to be possible joint overextension during injection. There were no other adverse events reported and no further issues with these control dogs throughout the study.

### **Veterinary Evaluation**

There were no significant ( $P > .05$ ) differences by investigator for any outcome variable; therefore, the data were pooled for further analysis. At baseline, there were no significant differences between the test and control groups in terms of veterinarian or owner scores. After treatment, veterinary orthopedic examination

scores for all parameters decreased over time in the stem cell group and, to a lesser extent, in the placebo group (Table 1).

The improvement in clinical scores was statistically significant in the stem cell group at all posttreatment evaluation times for lameness at walk and trot, pain on manipulation, and pain-free range of motion (Table 1). Functional disability, a highly subjective evaluation (Figure 1), added variance to the data that caused a lack of significance at later time points. Control animals did not significantly improve over time for lameness, pain on manipulation, or range of motion. Veterinary assessment revealed greater improvement from baseline for lameness at a trot, pain on manipulation, and range of motion in test animals compared with placebo controls (Figure 2). The combined scores for all parameters measured are also shown in Figure 2. There was no correlation between an animal's weight and its improvement score.

To determine which of the orthopedic examination parameters were most responsive to stem cell therapy and the timing of the responses, the effect size was calculated for each parameter at each time point. By this analysis, lameness at the trot, pain on manipulation, and range of motion were the most responsive, with large effect sizes (>0.8) at all posttreatment times. Comparison of average effect sizes by parameter are presented in Figure 3. The overall effect size was 1.34, which is considered a large effect in orthopedics.

### Owner Evaluation

Overall, owners with treated dogs evaluated their dogs to be more improved in the parameters scored, relative to owners with control dogs (Figure 4). Five dogs were eliminated as a result of multiple owner evaluations or compounding clinical conditions that made it impossible for owners to accurately evaluate their animals. Figure 4 demonstrates that treated dogs had a higher percentage of improvement overall (all scores combined) relative to control animals, although data did not reach statistical significance. Owners evaluated the dogs for the parameters that affected their dog most. Therefore, not all parameters were applicable to all dogs. In review of the 13 parameters scored on a 5-point scale, the control dogs had 1.9 parameters that improved by 2 or more points, whereas the test dogs had almost 4.7 parameters that improved by 2 or more points.

### DISCUSSION

Effective therapies for OA have been slow in developing, and many dogs continue to suffer with chronic pain associated with OA, even with multimodal treatment protocols. Results of this double-blind, placebo-controlled study demonstrate that AD-MSC therapy resulted in improved orthopedic examination scores as assessed by skilled veterinarians. Treated dogs' lameness, range of motion, and pain on manipulation, as well as their overall combined scores, significantly improved over time and improved relative to control animals. In this study, owner evaluation scores, although improving, did not reach significance. Circumstances created difficulty in interpretation of the owner data, including one household that introduced a new puppy that reportedly encouraged more spontaneous play on the part of a control dog and one test dog with a concurrent elbow condition that made isolation of the hip parameters difficult for the owner.

Results of this study again illustrate the value of systematically applied clinical scoring systems in detecting therapeutic effects. Effect size analysis, which was possible because of the double-blind, placebo-controlled design of the study, is particularly convincing. Although there are few reports in veterinary orthopedics with which to compare the present results, similar effect sizes were reported in a clinical trial of another intervention in orthopedic conditions in dogs.<sup>31</sup> It is also useful to note that the overall effect size for stem cell therapy under conditions described was 1.34, which is much higher than the aggregate effect size of 0.44 for glucosamine in humans with OA.<sup>32</sup>

The current study design employs a subjective numerical rating scale for veterinarians to assess degree of lameness. Although controversy exists as to how subjective scoring systems compare with objective gait analysis, subjective lameness score data have correlated well in cases of acute and chronic lameness of the stifle joint<sup>34-37</sup> and in dogs that underwent total hip replacement for OA.<sup>38</sup> Quinn and colleagues recently studied this question in detail and demonstrated that subjective scoring scales are not a replacement for force plate analysis.<sup>39</sup> However, subjective scoring systems are useful in clinical settings in which force plate analysis is impractical, such as the multicenter setting of this trial. The blinded nature of the study ensures

that veterinary bias is negligible; almost all dogs were evaluated by one veterinarian throughout all time points, and the scores reported for all dogs were based on solid examination findings. We have follow-up studies designed to evaluate the effects of AD-MSC therapy using force-plate analysis in a controlled environment.

This study was designed to evaluate the clinical effects of AD-MSC therapy on OA and not to determine the molecular mechanisms. However, many published in vitro and in vivo studies have explored these mechanisms. The immunomodulatory effects of BM-MSCs are well documented and represent one therapeutic mechanism in which AD-MSCs may function.<sup>8,40–42</sup> AD-MSC therapy can ameliorate severe graft-versus-host disease in people.<sup>43</sup> MSCs are well known to secrete cytokines and growth factors and may stimulate recovery in a paracrine manner.<sup>8,29</sup> Specifically, Ortiz and colleagues recently reported that BM-MSCs secrete interleukin-1 (IL-1) receptor antagonist (IL-1ra), which they determined to be the specific mechanism that reduced inflammation and fibrosis in a mouse model of lung injury.<sup>43</sup> IL-1 is known to play a significant role in joint disease and is believed to be high in the cytokine cascade in all animal species for which it has been studied.<sup>44</sup> Inhibiting IL-1 with IL-1ra has been shown to play a beneficial role in equine OA<sup>45–47</sup> and, therefore, is a likely mechanism by which AD-MSCs may mediate their effect in canine OA. Finally, cell-based tissue regeneration may play a role similar to that seen in the rabbit model of osteochondral defects.<sup>19</sup> One could imagine that the AD-MSCs may engraft in synovium or in cartilaginous lesions and either influence the local cells to differentiate into cartilage or the AD-MSCs themselves may differentiate into cartilage. Although the work by Nathan and associates demonstrates that new cartilage was formed,<sup>19</sup> other work reveals low levels of BM-MSC engraftment in a model of spinal cord injury.<sup>47</sup> Chopp and colleagues report that they have no evidence that the clinical benefit of BM-MSC therapy in the test rats was the result of differentiation but suggest a different hypothesis; that is, when these cells are placed in an environment of injury, they express cytokines and growth factors that promote repair or activate compensatory mechanisms and endogenous stem cells within the tissue.<sup>48</sup> These trophic mechanisms, rather than differentiation, are the current prevailing theory regarding the clinical benefits attributed to stem cell therapy.<sup>29,48</sup>

## CONCLUSION

Overall, dogs with OA of the coxofemoral joint that were treated with intraarticular injection of AD-MSCs demonstrated statistically significant improvement in lameness compared with a blinded, saline-injected control group and significant improvement over time from baseline. Indeed, three of these dogs had owners who were considering euthanasia because of their animal's pain and functional disability. These dogs are now living relatively pain free. The desired clinical outcome for a new treatment modality is better control of patient discomfort and increased functional ability. This multicenter study shows that intraarticular administration of adipose-derived stem and regenerative cell therapy decreases patient discomfort and increases patient functional ability.

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