








Original Article
Internal Medicine



Long-term treatment of allogeneic adipose-derived stem cells in a dog with rheumatoid arthritis

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Received: Mar 13, 2022

Revised: Jun 8, 2022

Accepted: Jun 17, 2022

Published online: Jul 4, 2022

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ABSTRACT

Background: Although there are growing demands for stem cell-based therapy for companion animals in various diseases, a few clinical trials have been reported. Moreover, most of them are the results from only one or a few times of stem cell injection.

Objectives: The aim of this study is to describe a long-term treatment with allogeneic adipose-derived stem cells (ASCs) in a dog with rheumatoid arthritis (RA), which is a rare canine disease.

Methods: The dog with RA received intravascular injection of allogeneic ASCs derived from two healthy donors once a month for 11 months. To assess therapeutic effects of ASCs, orthopedic examination and clinical evaluation was performed. Cytokines of tumor necrosis factor- α and interleukin-6 in the plasma were measured using ELISA analysis.

Results: Despite this repeated and long-term administration of allogeneic ASCs, there were no side effects such as immunorejection responses or cell toxicity. The orthopedic examination score for the dog decreased after ASCs treatment, and the clinical condition of the dog and owner's satisfaction were very good

Conclusions: Although ASCs has been suggested as one of the options for RA treatment because of its anti-inflammatory and immunosuppressive functions, it has never been used to treat RA in dogs. The present report describes a case of canine RA treated with allogeneic ASCs for long-term in which the dog showed clinical improvement without adverse effects.

Keywords: Dog; immune-mediated disease; immunomodulation; mesenchymal stem cell; rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA), which is a chronic, noninfectious, and erosive polyarthritis, is uncommon in dogs and resembles the disease in humans [1]. In dogs, RA typically affects the stifles, carpi, tarsi, and digits of small-breed, middle-aged dogs, and is characterized clinically by progressive lameness [1,2]. Although the cause of RA is unknown in both humans and animals, it is clear that the disease is related to humoral and cell-mediated

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Conflict of Interest

The authors declare no conflicts of interest.

Funding

This work was supported by the National
Research Foundation of Korea (NRF) grant
funded by the Korean government (MSIT) (No.
2020R1A2C102143).

immunity, which produce autoantibodies to immunoglobins (Igs) in response to an unknown stimulus [1,3,4]. Joint inflammation in RA is mediated by the influx of immune cells into the synovial joint space, and the joint is swollen with excess synovial fluid comprising activated synovial fibroblasts, lymphocytes, macrophages, and neutrophils, which secrete pro-inflammatory mediators such as cytokines, chemokines, and prostaglandins [5]. The lesions of RA are typically symmetrical and severe, including synovial villus hypertrophy, synoviocyte proliferation, fibrin deposition, synovial necrosis, lymphoplasmacytic synovitis, and destruction of cartilage and subchondral bone; subsequently, degenerated articular cartilage is replaced with granulation tissue (pannus) [1,2]. The diagnosis of canine RA is difficult. In addition to clinical signs, radiological evidence of joint erosion, serological analyses for rheumatoid factor (RF) or autoantibodies, laboratory analysis of synovial fluid, and joint biopsy may assist in diagnosis [2,3].

Traditionally, the treatment of RA in humans and dogs is directed to control pain and retard the erosive progression of the disease [2,6]. In dogs, immunosuppression with prednisone and mycophenolate has been recommended [2]. In humans, therapeutic drugs to impede the inflammation in RA can be classified into four categories: non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, non-biological disease-modifying anti-rheumatic drugs (DMARDs), and biologic DMARDs [7,8]. Although conventional drug therapies for RA can ameliorate inflammation and symptoms of the disease, a substantial number of patients with RA remain resistant to these treatments. Moreover, numerous side effects limit its long-term use, leading to the need to explore the development of new therapeutic strategies that can be more effective, safe, and tissue-regenerative [9,10]. In this regard, cell therapy with mesenchymal stem cells (MSCs) has recently become the most attractive new approach to address unresolved treatment issues in RA [7]. MSCs are multipotent cells with the capacity for self-renewal and regeneration of tissues and organs, and moreover, their anti-inflammatory, immunomodulatory, immunosuppressive, angiogenic, and antioxidant properties are in the spotlight as a therapeutic potential for clinical application of RA [7,11].

Although there are growing medical demands for an alternative therapy in rebellious diseases, a few clinical trials of stem cell therapy have been reported in veterinary practice. Moreover, most of them are the results from only one or a few times of stem cell injection. We clinically applied allogeneic adipose-derived stem cells (ASCs) to dogs with RA. The dog was treated with allogeneic ASCs derived from the abdominal fat of healthy donors once a month for 11 months without any other medications. It is the first time to use allogeneic ASCs for long-term in a dog with RA, which is a rare disease in veterinary medicine.

MATERIALS AND METHODS

History and clinical findings

A 5-year-old female Maltese (2 kg) presented to a veterinary clinic with lameness. On radiographic examination, the carpal, tarsal, elbow, and stifle joints were swollen with crepitus because of severe inflammation. Radiographic images showed irregular radiopaque and radiolucent areas representing inflammation and bone lysis, respectively (**Fig. 1**). Numerous non-degenerative neutrophils were found in the synovial fluid. The dog had showed shifting lameness gradually progressing from the hind limbs to the fore limbs. A joint biopsy was performed about 4 months after the first visit. Most of the articular cartilage was replaced with granulation tissue (pannus) (**Fig. 2**). The shifting lameness lasted for

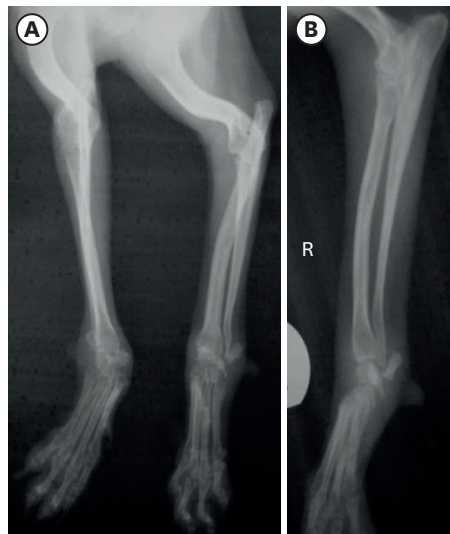


Fig. 1. Radiograph of the forelimbs at the first visit of the hospital. (A) The joints are swollen because of severe inflammation in tarsal and elbow joints. There are irregular radiolucent areas representing bone lysis. (B) Lateral radiographic view of the right forelimb. Joint inflammation and bone lysis are obvious.

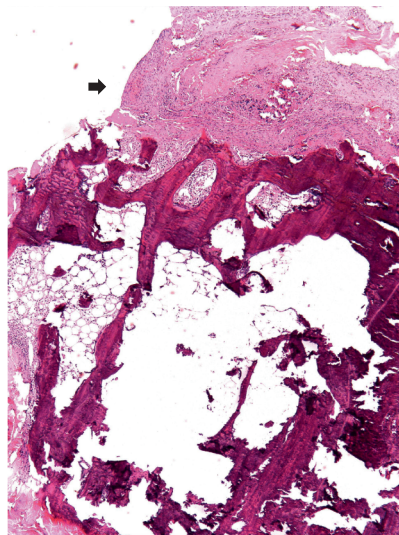


Fig. 2. The joint biopsy of the dog. The articular cartilage has been replaced with granulation tissue (pannus, arrow) (hematoxylin & eosin, scale bar = 100 μ m).

1-2 years, and finally, the dog became unable to walk due to severe osteopenia following pathologic fractures with angular deformation and ankylosis (**Fig. 3**). Consequently, the dog was diagnosed with RA based on clinical examination, synovial fluid analysis, and joint biopsy. The owner agreed with stem cell therapy because it was an attractive option already proved to be safe and have therapeutic effects in human RA patients. The dog intravenously (IV) received $5 - 10 \times 10^6$ cells of allogeneic ASCs, derived from abdominal fat of healthy donors (5-month-old mixed dog and 6-month-old Maltese dog), once a month for 11 months without any other medications. These allogeneic ASCs were not mixed, but ASCs from the mixed dog were injected for the first five times, and then ASCs from the Maltese dog was used for the rest of the period. Despite repeated injection of allogeneic ASCs, the dog did

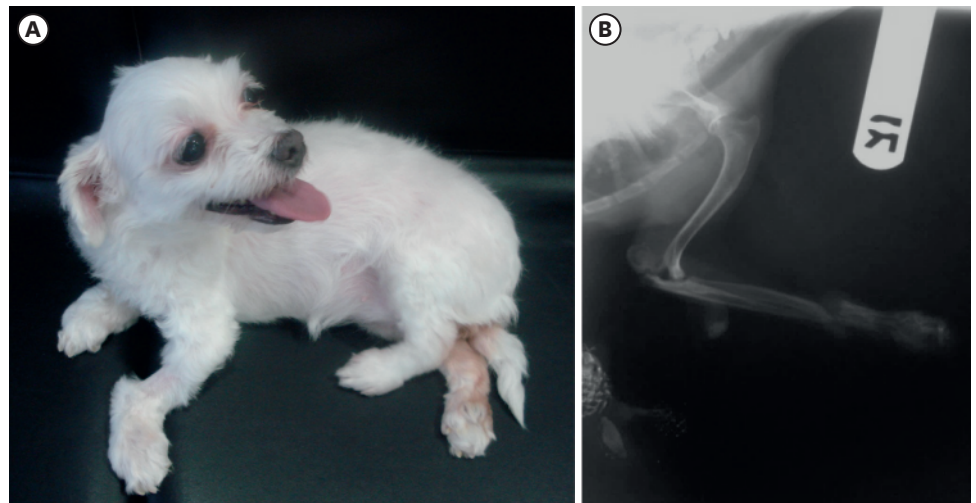


Fig. 3. The dog about a year after the first visit. (A) The dog is not able to walk due to severe angular deformation. Note severely deformed carpi, elbow, tarsi, stifle, and coccygeal joints of the dog. (B) Radiograph of the right forelimb. There are severe osteopenia following multiple pathologic fractures with angular deformation and ankylosis.

not show any side effects, such as cell toxicity or immunorejection responses. The dog died within a month after the last treatment of ASCs, and glomerular amyloidosis of the kidney was revealed at necropsy (**Fig. 4**).

Isolation, culture and characterization of ASCs

Abdominal fat was obtained from two healthy dogs (5-month-old mixed dog and 6-month-old Maltese dog) during ovariohysterectomy. The tissue was extensively washed with equal volumes of Dulbecco's phosphate-buffered saline (PBS), and then digested with 2 mg/mL collagenase type I (Worthington Biochemical, USA) at 37°C for 30 min in a shaking incubator. Digested tissue was filtered through a 70 µm nylon cell strainer to separate the dissociated cells from the undigested tissue. After centrifugation of the digested tissue, the pelleted stromal vascular fraction was collected carefully, washed with PBS, and then seeded onto culture dishes in a complete medium consisting of low-glucose Dulbecco's Modified Eagle Medium (Gibco, USA) containing 10% fetal bovine serum (Gibco) and 1% antibiotic/antimycotic solution (Gibco). Cells were kept at 37°C and 5% CO₂ in humidified incubators.

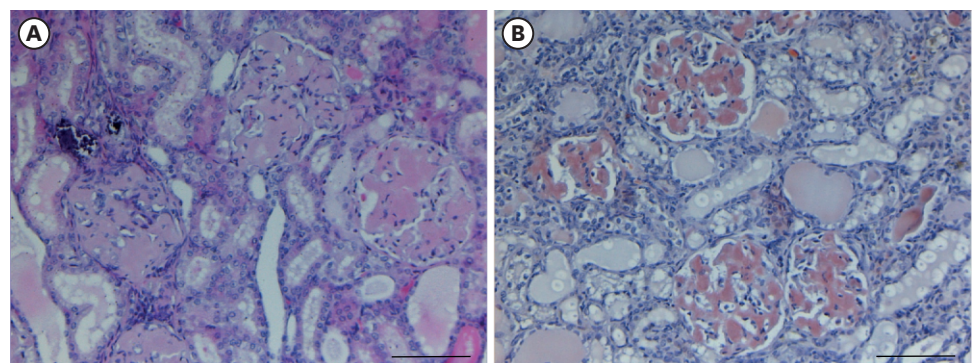


Fig. 4. Microscopic examination of the kidney of the dog. (A) Glomeruli are diffusely and notably expanded by pale eosinophilic homogenous hyalinized deposits (hematoxylin & eosin). (B) Amyloid in the glomeruli stains with Congo red staining (scale bar = 100 µm).

Table 1. Modified veterinary orthopedic examination assessment score sheet for clinical evaluation

Score	Pain on manipulation	Range of motion	Functional disability	Vitality assessment
1	No pain	No limitation	Normal activity	Excellent
2	Mild pain	Stiffness only at full range of motion	Stiff, dog has noticeable difficulty walking or running	Good
3	Moderate pain	Stiffness at less than full range of motion	Very stiff, dog does not want to walk or run	Fair
4	Severe pain	Stiffness at any attempt to manipulate joint	Dog must be helped up, and cannot walk	Poor
5			Dog cannot use limbs, and not stand up	Bad

Table 2. Standard questionnaire including 5 parameters to evaluate the owner satisfaction

Score	Stiffness in the morning	Stiffness at the end of the day	Willingness to play	Condition	Energy level
1	Absent	Absent	High	Good	Good
2	Mild	Mild	Intermediate	Fair	Fair
3	Moderate	Moderate	Low	Poor	Poor
4	Severe	Severe	Absent	Bad	Bad

Following incubation, the culture dishes were washed with PBS to remove non-adherent cells and red blood cells, and media change was done every two or three days. For characterization of ASCs using flow cytometry, the third passage (P3) expansion of ASCs was detached from the culture dish using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and washed with PBS. Cells were then suspended at a concentration of 5×10^5 cells in 200 μ L PBS and incubated for 20 min at 4°C with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies as follows: CD13, CD29, CD31, CD34, CD44, CD45, and CD117 (BD Pharmingen, USA). Cells were washed with PBS and then analyzed using flow cytometry (FACSARIA; BD Biosciences, USA). FITC- or PE-conjugated mouse IgGs were used as negative controls.

Enzyme-linked immunosorbent assay (ELISA) analysis

Whole blood samples were collected from the cephalic vein of the dog in EDTA-treated tubes, right before IV injection of ASCs once a month. Plasma was collected by centrifugation of blood, and tumor necrosis factor (TNF)- α and interleukin (IL)-6 in plasma were measured using ELISA (R&D Systems, USA) according to the manufacturer's instructions.

Clinical evaluation of the dog by the veterinarian and the owner

Physical examination, including pain on manipulation, joint mobility, functional ability, and vitality assessment, was performed by a veterinarian according to a modified version of published criteria based on veterinary orthopaedic examination assessment using a numerical rating scale (**Table 1**) [12]. The owner was asked to complete a standard questionnaire to evaluate the clinical changes in the dog after all ASC treatment using a numerical rating scale, which included the following five parameters: stiffness in the morning, stiffness at the end of day, willingness to play, condition, and energy level (**Table 2**) [13].

RESULTS

Characterization of canine-ASCs by flow cytometry

Flow cytometry analysis showed that canine-ASCs (P3) from the abdominal fat of healthy donor dogs expressed high levels of stromal cell-associated markers, including CD13, CD29, and CD44. Hematopoietic stem cell-associated markers (CD34, CD45, and CD117) and endothelial cell-associated marker (CD31) were not expressed (**Fig. 5**).

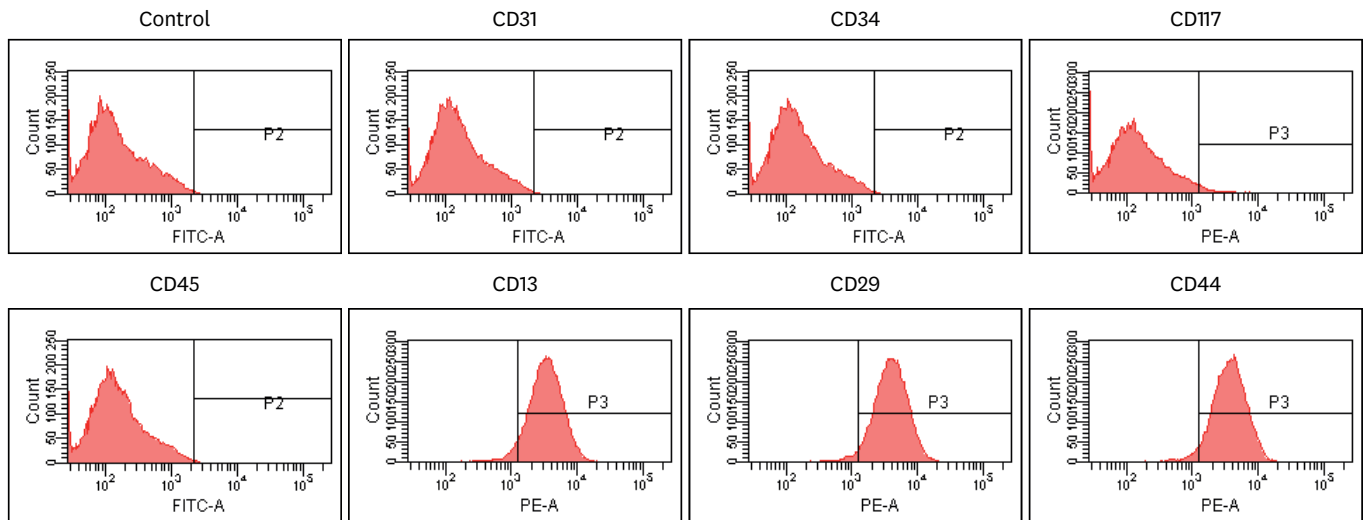


Fig. 5. Flow cytometry analysis of canine-ASCs from a healthy donor dog. Stromal cell-associated markers (CD13, CD29, CD44) are highly expressed, and hematopoietic stem cell-associated markers (CD34, CD45, CD117) and endothelial cell-associated marker (CD31) are negative. ASC, adipose-derived stem cell.

TNF- α and IL-6 expression in plasma of the dog after IV injection of ASCs

The plasma was collected from the dog once a month right before the IV injection of ASCs. The expression of TNF- α in the plasma of the dog was very low, except at 5 months after the first injection of ASCs (**Fig. 6A**). IL-6 expression in the plasma was also very low, except at 4 and 5 months after the first injection of ASCs (**Fig. 6B**).

Clinical evaluation scores for the dog after ASC treatment by the veterinarian and the owner

Veterinary orthopaedic examination scores revealed that the dog was improved in the range of motion, functional disability, and subjective assessment after ASC treatment (**Table 3**). In the owner's evaluation, the scores for all parameters also decreased after ASC treatment (**Table 4**). The owner stated that the dog was more improved in all parameters, including stiffness in the morning and at the end of the day, willingness to play, condition, and energy level.

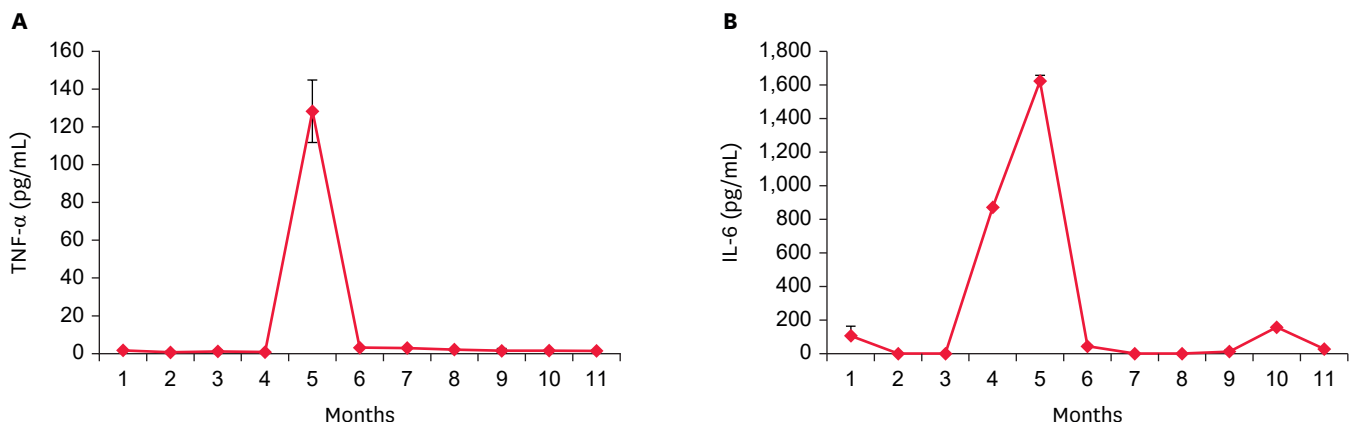


Fig. 6. Inflammatory cytokine expression in the plasma of the dog. (A) TNF- α expression is very low except at 5 months. (B) IL-6 expression is also very low except at 4 and 5 months. TNF, tumor necrosis factor; IL, interleukin.

Table 3. Veterinary orthopedic examination scores evaluated by the veterinarian

Parameter	Pre-treatment	Post-treatment
Pain on manipulation	1	1
Range of motion	4	3
Functional disability	5	4
Vitality assessment	5	2

Table 4. Clinical examination scores evaluated by the owner

Parameter	Pre-treatment	Post-treatment
Stiffness in the morning	4	3
Stiffness at the end of the day	4	3
Willingness to play	4	2
Condition	4	1
Energy level	4	1

DISCUSSION

RA in dogs is uncommon, and its diagnostic criteria are referenced for the diagnosis of RA in humans. Clinical signs, including pain, stiffness, and swelling in multiple joints due to synovitis, and radiological evidence of joint erosion are typical indications for RA. The presence of autoantibodies such as RF, anti-cyclic citrullinated protein (CCP) antibody, and anti-nuclear antibodies (ANA), or an increase in C-reactive protein (CRP) level or erythrocyte sedimentation rate (ESR) suggest a diagnosis of RA in humans [14]. However, all serological tests of all RA patients in both humans and dogs cannot be expected to produce positive results as serum autoantibody levels can be elevated in other inflammatory diseases [2,14]. Positive tests for RF or autoantibodies support a diagnosis of RA, but clinical signs, radiographic, and histopathologic changes of joints are much more important to make a definitive diagnosis of RA in dogs [2,15]. In the present case, a definitive diagnosis was made based on clinical signs, radiographic images of joint erosion, synovial fluid analysis, and histopathological findings of pannus formation in the joint.

Chronic inflammation in RA is characterized by altered innate and adaptive immunity, including immune responses against autoantigens, dysregulated cytokine networks associated with pro-inflammatory cytokines (such as TNF- α , IL-6, IL-1, and IL-17) and immune complex-mediated complement, and increased osteoclast activity, which mediates excessive bone resorption and chondrocyte activation [16-18]. However, there are a few data regarding cytokine profiles in canine RA, which is still controversial. Carter et al. reported that IL-6 activity is increased in the synovial fluid of canine joints affected by RA rather than TNF- α and IL-1 [3]. Hegemann et al. [19] found that IL-1 β mRNA is highly expressed in the synovial fluid of dogs with immune-mediated arthritis, including RA, whereas TNF- α and IL-6 mRNA expression were not different compared with dogs with osteoarthritis by cranial cruciate ligament rupture. In the present case, both TNF- α and IL-6 in the plasma of the dog were detected to be extremely low, with a temporary increase at 5 months and 4–5 months, respectively, after ASC administration. Although these data may not accurately reflect the condition of arthritis since they were from plasma and not synovial fluid, we assumed that long-term chronic inflammation in the dog may cause depletion of cytokines from inflammatory cells, and no clinical changes such as further complaints of pain were detected at the time of temporary increase in cytokines. Further analyses could be attempted to clarify the therapeutic effect of ASCs for RA, but it was very limited because the dog was a clinical patient.

At present, various clinical trials using MSC-based therapies have been conducted for the treatment of immune-mediated disorders involving graft vs. host disease, inflammatory bowel disease, multiple sclerosis, systemic lupus erythematosus, type I and II diabetes, primary Sjogren's syndrome, ankylosing spondylitis, and RA in human [7,20]. In veterinary medicine, the medical demands for MSC-based therapy for companion animals have also been rapidly growing in various diseases. Clinical trials using stem cells for companion dogs and cats have been reported for the treatment of cardiovascular, neurologic, dermatologic, gastrointestinal, and musculoskeletal diseases, as well as cancers [9,21]. In particular, ASCs can be easily acquired in large quantities using a minimally invasive technique, which makes them easier to be utilized than bone marrow-derived stem cells [22]. In humans, based on clinical studies using IV injection of autologous or allogeneic MSCs, encouraging results in terms of safety and therapeutic efficacy have been observed in refractory patients with a long history of RA [7]. In clinical trials, administration of allogeneic MSCs in RA patients was well-tolerated with no adverse events and no evidence of cell quantity-related toxicity [23,24]. Recently, a prospective phase I/II study reported that umbilical cord-MSCs plus DMARDs therapy decreased the levels of CRP, RF, anti-ESR, and anti-CCP in RA patients, and these beneficial effects were observed in the long-term, for up to 3 years [25]. In this case, allogeneic ASCs from two different canine donors were injected intravascularly for 11 months, and no adverse effects were observed. Especially, the owner's satisfaction for the clinical improvement of the dog was very good, and this is also the reason the owner continued ASC treatment for almost a year. Unfortunately, the dog died due to renal amyloidosis, which is often associated with chronic inflammatory disease and also known as one of the major complications of RA in humans [26]. Consequently, stem cell injection to the dog in the present case may not prevent the progression of RA or the occurrence of complications, but it may improve the quality of life by reducing pain or inflammation.

The present report describes a case of canine RA treated with IV injection of allogeneic ASCs from two donors, once a month for 11 months. Although the dog died due to glomerular amyloidosis as a complication of RA, clinical signs were significantly improved without adverse effects during the treatment. This repeated and long-term administration of allogeneic MSCs in a dog with RA is the first trial in veterinary medicine and suggests that ASC treatment can substitute or assist currently used treatments in RA or immune-mediated diseases.

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