Allogeneic adipose-derived mesenchymal stem cell therapy in dogs with refractory atopic dermatitis: clinical efficacy and safety

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Canine atopic dermatitis (AD) is a common skin disease with a 10–15 per cent prevalence. Current treatments vary in their efficacy and safety. The immunomodulatory properties of mesenchymal stem cells (MSCs) make them a promising alternative treatment. The aim of this study was to evaluate the therapeutic efficacy and safety of allogeneic canine adipose MSCs (cAd-MSCs) in dogs with refractory AD. Twenty-six dogs, suffering from AD for at least 12 months, not responding to conventional therapy, received an intravenous dose of 1.5×10^6 cAd-MSCs/kg bodyweight. Clinical signs, haematological and biochemistry profiles, and AD severity were assessed in a six-month follow-up using a validated scoring system (Canine Atopic Dermatitis Extent and Severity Index, version 4 (CADESI-04)). The degree of pruritus was quantified using a validated visual analogue scale, and also owner's global assessment of treatment efficacy. Twenty-two animals completed the study. Pruritus and CADESI-04 scores decreased significantly after one week or month of treatment, respectively, and remained stable for six months. Owner's global assessment score was 2.15±1.15 for all the animals in the study. In conclusion, systemic administration of allogeneic cAd-MSCs appeared to be a simple therapy with positive outcome in the remission of clinical signs for AD refractory to conventional medications, for at least six months and with no adverse events.

Introduction

Atopic dermatitis (AD) is one of the most common skin diseases in dogs, with a prevalence ranging from 10 to 15 per cent of the canine population.[1–3] Canine AD is a multifaceted inflammatory disease resulting from complex interactions between environmental factors (such as ingestion of food and allergens) and genetic predisposition (ie, filaggrin mutations), which can modify not only the skin barrier but also the immunological response of the patient.[2 4–6]

The inflammatory reaction is caused by biphasic T cell polarisation. The initial acute phase is marked by a strong T helper (Th)2 profile response with specifically produced cytokines such as interleukin (IL)-4, IL-13 and IL-31, among others.[2 4 5 7 8] IL-4, IL-5 and IL-13 activate recruitment of eosinophils to the inflammatory site, in addition to B cell proliferation and differentiation, promoting the production of IgE antibodies. IL-31 is actively involved in the production of pruritus.[9 10]

In chronic lesions, there is a lower expression of these Th2 cytokines, whereas an increase of Th1 profile cytokines, such as IL-2 and γ-interferon, is observed.[4 7] Canine AD disease typically begins as a Th2 polarisation and progresses to become a chronic disease with a mixed Th2/Th1 profile.[7 10]

Current canine AD treatment protocols, among others, include the use of different types of immunomodulatory drugs. However, a percentage of dogs do not respond to standard treatments or relapse after a while.[1 11 12]

Mesenchymal stem cells (MSCs) are multipotent stem cells with the capacity to differentiate into diverse cell lineages. They are also capable of different

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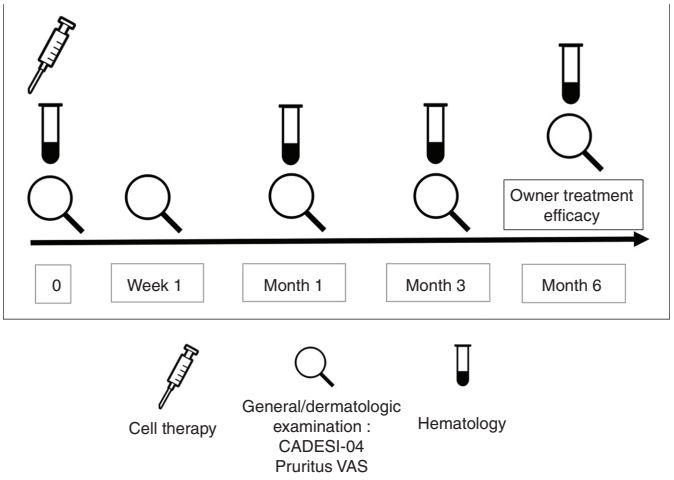

Figure 1 Experimental design. Animals were evaluated at baseline (0), at 1 week and at 1, 3 and 6 months after cell therapy. CADESI-04, Canine Atopic Dermatitis Extent and Severity Index, version 4; VAS, visual analogue scale.

bioactive molecules with trophic, paracrine and immunomodulatory functions.[13–16]

MSCs express low levels of major histocompatibility complex class I (MHC-I) but lack expression of MHC-II surface molecules. They exhibit immunosuppressive effects on MHC-mismatched lymphocyte proliferation, and these cells can also inhibit naive, memory and activated T cells, B cells, natural killer cells and dendritic cells.[16 17]

Their low immunogenicity and their immunomodulatory potential allow their allogeneic use, which makes them a promising new treatment for severe refractory autoimmune diseases.[17–22] They have been extensively studied as a cellular therapy for different pathological conditions, using the dog as an animal model.[19 20 23–26] There are currently many studies in both animals and human beings that demonstrate the efficacy of MSCs in AD.[27 28] The interest in their allogeneic clinical use is due to existence of limiting factors, such as age, medications and concomitant diseases, which may have an impact on the quality and immunomodulatory capacity of autologous MSCs.[29]

The aim of this study was to evaluate the safety and therapeutic long-term results (six months of follow-up) of a single dose of allogeneic canine adipose MSCs (cAd-MSCs) in dogs with refractory AD.

Materials and methods

This was an uncontrolled open-label study. Written informed consent was obtained from all owners.

Animals

Inclusion criteria

Twenty-six client-owned dogs were included in this study. All the animals have been suffering from chronic AD for at least 12 months, before recruitment. They were selected based on their clinical histories and fulfilment of at least five of eight published criteria.[30] All of them were refractory to conventional treatments (diet and nutritional supplements, topical treatments, antimicrobial therapy, allergen-specific immunotherapy, immunomodulatory medication), and no viable therapeutic alternatives were available. Other pathologies (metabolic diseases, ectoparasitic and infectious dermatoses) were excluded by appropriate complementary diagnostic tests.

Dogs with evidence of neoplasia or medical conditions that could have affected immune function were not included.

Table 1 Patient baseline values (mean±sd)

N	26
Age (years±sd)	6.3±2.4
Male, n (%)	11 (42)
Female, n (%)	15 (58)
Weight (kg)	19.6±8.1
Atopic dermatitis duration (years±sd)	2.54±1
CADESI-04 (0–180)	35.4±21.4
VAS pruritus score (0–3)	8.36±1

CADESI-04, Canine Atopic Dermatitis Extent and Severity Index, version 4; VAS, visual analogue scale.

Prohibited and allowed medications and therapies

Local or systemic anti-inflammatory or immunomodulatory medications were withdrawn two weeks before cell therapy and were suspended during the six-month follow-up period. When additional treatment was necessary for animal welfare reasons, the animal was withdrawn from the study. The same diet, flea and hygiene protocol was maintained during the study. Dogs were permitted to receive antimicrobial or antifungal therapy in the follow-up under the specialist criterion, based on clinical and complementary microbiological tests.

Clinical evaluation

The study design is illustrated in figure 1. Dogs were evaluated before cell therapy administration (table 1) at 1 week and at 1, 3 and 6 months after treatment. A complete physical and dermatological examination was performed during all follow-up appointments by the same clinician. Haematological and serum biochemistry profiles were obtained the day of treatment and at 1, 3 and 6 months.

Clinical signs and severity of AD were assessed using a previously validated scoring system (Canine Atopic Dermatitis Extent and Severity Index, version 4; CADESI-04) ranging from 0 to 180.[31] Owner pruritus degree was quantified using a validated visual analogue numerical scale (VAS) ranging from 0 to 3.[32] Owner's global assessment of treatment efficacy was also evaluated at the end of follow-up using a 0–3 scale (0=unsatisfactory; 1=satisfactory; 2=very satisfactory; 3=excellent).

To minimise the possible effects of seasonality on clinical signs, cell therapy was administered to all animals within a six-week period.

Table 2 Demographic, clinical data and evolution of dogs included in the study

Dog	Sex*	Age†	Breed ‡	Weight §	AD history ¶	Day 0		1 week		1 month		3 months		6 months		Owner satisfaction **
						CAD-4**	VAS **	CAD-4**	VAS **	CAD-4**	VAS **	CAD –4**	VAS **	CAD-4**	VAS **	
1	SF	8	EB	33	2	35	8	19	3	12	1	9	0	13	1	3
2	SF	9	W	11	1,5	82	9	64	7	10	3	9	1	9	1	2
3	M	1,5	GR	30	1	14	7	8	9	14	4	14	5	14	6	1
4	SF	7	GS	30	2	29	7	4	3	4	2	2	1	2	0	3
5	SF	6	SWD	11	3	29	6	14	6	4	3	4	3	–	–	0
6	F	6	FB	15	2	58	9	30	7	9	2	5	1	5	0	3
7	M	5	GR	32	2,5	49	9	28	4	14	3	6	2	6	0	3
8	SF	9	FB	16	1,5	43	8	40	7	12	4	8	3	8	3	2
9	CM	5	W	11	1	74	10	72	9	60	9	–	–	–	–	0
10	SF	3	EB	22	1	31	7	16	4	6	2	4	1	4	0	3
11	F	5	FB	12	1,5	46	8	35	7	16	61	16	5	–	–	1
12	CM	9	GR	35	4	38	9	16	5	17	2	23	3	25	5	1
13	M	6	C	25	3	71	10	68	9	20	8	20	6	15	5	3
14	F	5	GR	29	3	18	7	17	9	14	6	14	6	–	–	0
15	SF	7	W	12	2	19	7	13	4	10	4	8	2	6	1	3
16	F	7	FB	18	3	26	9	12	8	8	9	6	5	6	3	2
17	M	3	S	25	2	12	9	7	8	5	6	5	2	5	2	3
18	F	10	W	12	4	40	9	35	7	16	5	6	4	4	1	3
19	M	2	MB	25	1	14	9	12	6	1	3	0	2	0	0	3
20	SF	4	FB	15	3	34	7	21	4	4	3	2	1	0	1	3
21	M	8	EB	25	4	24	9	19	9	8	6	6	4	3	1	2
22	M	5	MB	20	2	84	10	84	10	65	8	74	7	82	8	0
23	F	8	W	11	4	18	9	16	7	9	4	8	1	3	0	3
24	SF	10	FB	12	4	18	8	8	5	6	2	2	2	0	1	3
25	M	6	FB	15	4	13	6	11	3	4	1	0	0	0	0	3
26	SF	10	W	10	4	41	9	28	3	12	3	6	2	4	2	3

*Sex: CM, castrated male; F, female; FS, spayed female; M, male.
†Age in years.
‡Breed: C, chow chow; EB, English bulldog; FB, French bulldog; GR, golden retriever; GS, German shepherd dog; MB, mixed breed; S, Stanford; SWD, Spanish water dog; W, West Highland white terrier.
§Weight in kilograms.
¶AD (atopic dermatitis) history in years.
**Clinical evaluation: CADESI-04 (CAD-4) (0–180) and pruritus VAS score (VAS) (0–9). Owner satisfaction (0–3).
CADESI-04, Canine Atopic Dermatitis Extent and Severity Index, version 4; VAS, visual analogue scale.

Isolation, culture and characterisation of cAd-MSCs

cAd-MSCs were obtained and characterised as described in previous publications.[19 20] A brief description of the procedure detailed in those publications is as follows: adipose tissue was digested with collagenase type II (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), filtered and centrifuged to obtain the cell pellet. Primary cultures were carried out with Dulbecco's modified Eagle's medium (DMEM) containing 10 per cent fetal bovine serum, 2.5 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml fungizone (all from Sigma-Aldrich, Merck KGaA). Cells were detached when confluence was over 80 per cent and subcultured at a concentration of 10^4 cells/cm^2 for continued passaging. The remaining cells were cryopreserved and stored in liquid nitrogen. All experiments and in vivo implantation were conducted at passage 2.

Flow cytometry analysis

Fluorescence-activated flow cytometry (FC) was used to characterise cAd-MSCs at passage 2 as previously described,[19 20] against CD29, CD44, CD73, CD90 and STRO-1 (R&D Systems, Minneapolis, USA), CD11/18, CD34 and CD45 (Miltenyi Biotec SL, Germany), and MHC-II (BD Pharmingen, Becton Dickinson, New Jersey, USA).

In vitro multilineage cell differentiation

To assess multipotentiality, cAd-MSCs at passage 2 were differentiated along adipogenic, osteogenic and chondrogenic lineages according to standard protocols, as previously described.[19 20]

Immunomodulatory potential

For this purpose, the cAd-MSC capacity of proliferation inhibition of allogenic peripheral blood mononuclear cells (PBMCs) was evaluated.[33]

Briefly, PBMCs were separated from the whole blood of seven healthy dogs using Ficoll-Hypaque density gradient centrifugation and stained with 4 µM 5-chloromethylfluorescein diacetate (CMFDA, CellTracker Green Kit C2925, Thermo Fisher Scientific, Waltham, Massachusetts, USA). cAd-MSCs at passage 2, previously inactivated with mitomycin C for three hours, were cocultured with PBMCs at a proportion of 1:5. Concanavalin A (ConA; Sigma-Aldrich, Merck KGaA) was used as mitogen at a final concentration of 5 µg/ml. The following experimental groups were made in

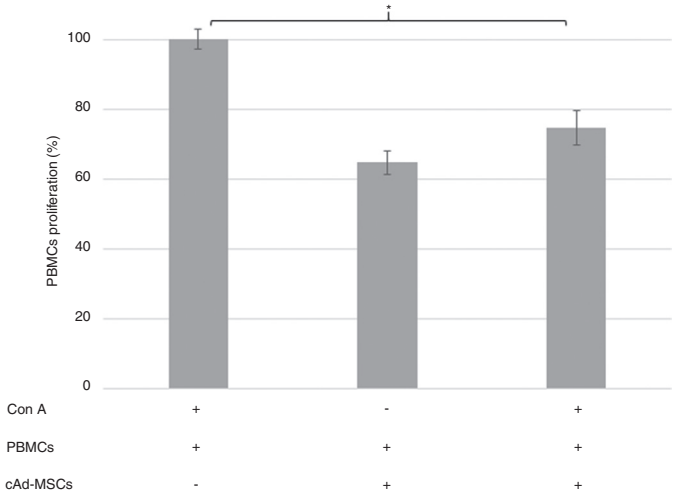

Figure 2 Suppression of canine peripheral blood mononuclear cell (PBMC) proliferation cocultured with canine adipose mesenchymal stem cells (cAd-MSCs) for 72 hours and stimulated with concanavalin A (ConA). Values are mean±sd. Asterisk (*) indicates a statistically significant difference (P<0.05).

triplicate: PBMCs and ConA; PBMCs and cAd-MSCs; and PBMC, cAd-MSCs and ConA. Cells were incubated for 72 hours at 37°C and 5 per cent CO_2, and then analysed by means of FC (Beckman Coulter, California, USA). For comparison, lymphocytes stimulated with ConA were set to 100 per cent proliferation. FC data were analysed using FlowJo cytometry software (FlowJo, Ashland, Oregon, USA).

Karyotype

cAd-MSCs at passage 2 were karyotyped as previously described.[34] cAd-MSCs were cultured until semiconfluence. Then, cells were harvested and treated with 0.07 µg/ml of Colcemid (Thermo Fisher Scientific) for 30 minutes to arrest mitotic cells in metaphase. Subsequently, pelleted cells were resuspended in a hypotonic solution (0.075 M potassium chloride solution) for five minutes to swell cells. cAd-MSCs were then fixed in cold methanol glacial acetic acid (3:1) and washed three times to ensure complete removal of cytoplasmic debris. Afterwards, they were stained in 2 per cent Giemsa and analysed with ordinary bright-field microscopy. Analyses included scanning all slides, counting a minimum of 20 metaphases, analysing a minimum of seven metaphases and karyotyping a minimum of two metaphases.

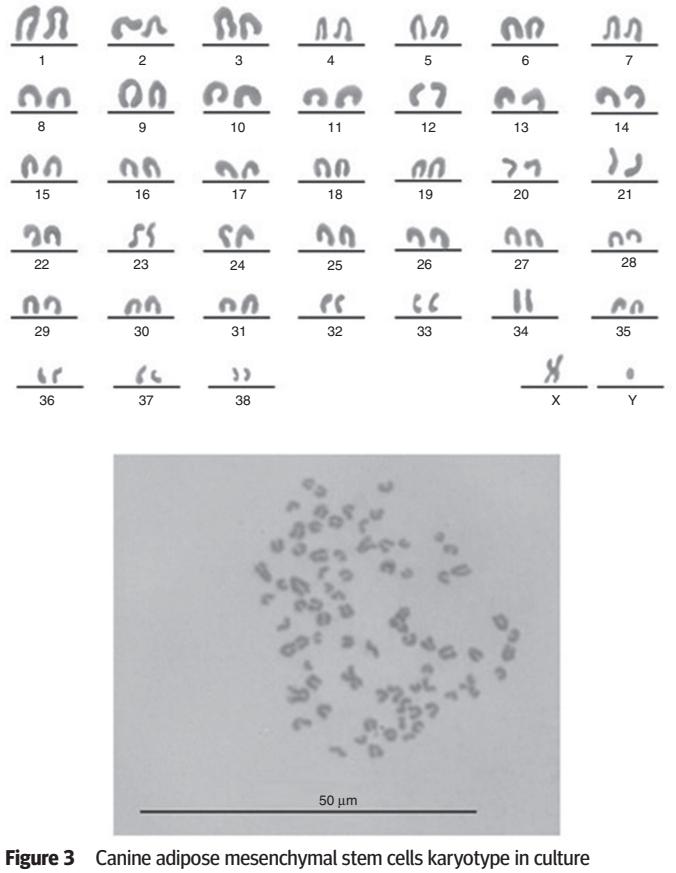

Figure 3 Canine adipose mesenchymal stem cells karyotype in culture passage 2.

Treatment protocol

Twenty-six dogs received one allogeneic dose of 1.5×10^6 cAd-MSCs/kg bodyweight in 2-ml DMEM diluted in 50-ml physiological saline serum and administered over 30 minutes through a peripheral intravenous cannula. The infusion was controlled by a veterinary surgeon. The dogs were monitored for 60 minutes following infusion and before being discharged.

Statistical analysis

All values are expressed as mean±sd. Analysis of variance (ANOVA) with post-hoc Dunnett test was used to compare CADESI-04 at different times. Owner pruritus score and owner's global assessment of treatment efficacy analysis was performed using the Wilcoxon test. Immunomodulatory potential was assessed by comparing the percentage of PBMC proliferation between groups with an ANOVA post-Bonferroni *t* test.

Differences were considered significant when P<0.05. All analyses were carried out using SPSS 16 (IBM Corporation, Armonk, New York, USA).

Results

Animals

Twenty-six dogs of different breeds, 11 males and 15 females, with ages ranging from 1.5 to 10 years old (6.3±2.4 years) and weighing 11–35 kg (19.6±8.1 kg), were enrolled in the study (mean±sd for both variables, age and weight). All animals showed a history compatible with AD for at least 12 months (mean 2.54±1.0 years) and failed to respond to or had been unable to tolerate AD conventional therapy (table 2).

Characterisation of allogeneic cAd-MSCs

FC analysis

The profiles of cAd-MSCs revealed a homogeneous cell population, positive to mesenchymal markers (CD29, CD44, CD73, CD90 and STRO-1) and negative for the expression of haematopoietic markers (CD11/18, CD34, CD45 and MHC-II).

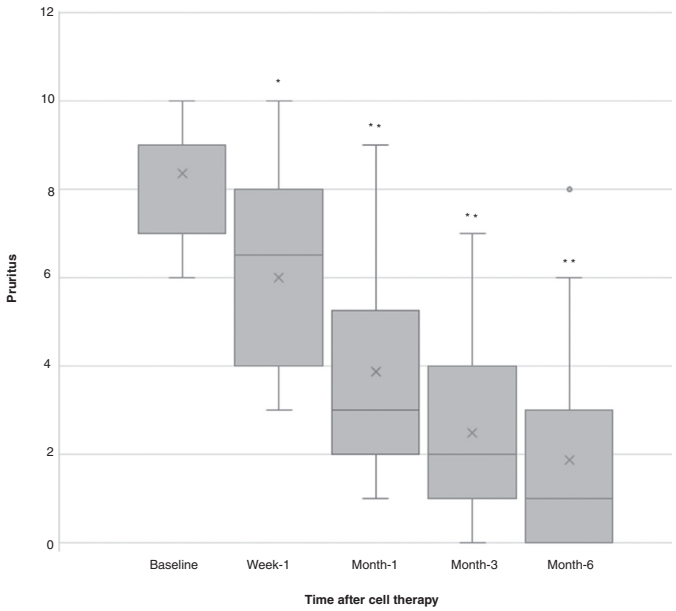

Figure 4 Pruritus visual analogue scale scores. Mean pruritus score improved significantly one week after cell therapy and remained stable until the end of follow-up. Data represent mean±sd; *P<0.05 and **P<0.001 versus baseline.

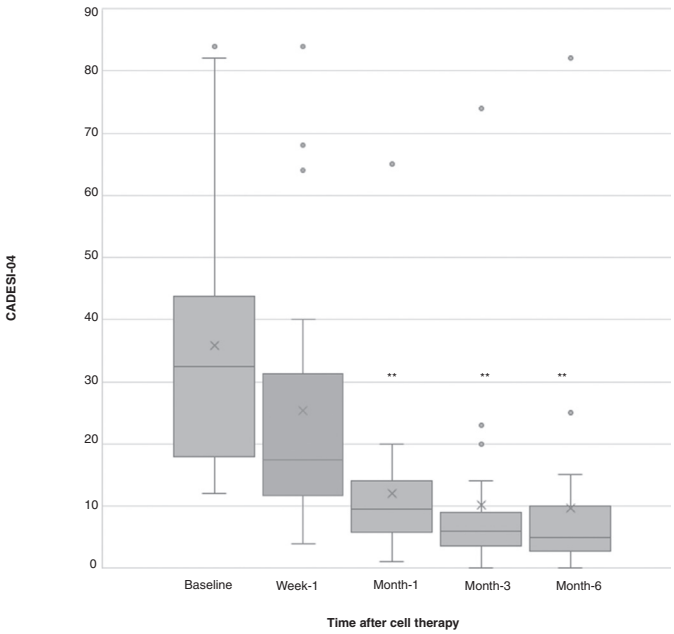

Figure 5 CADESI-04 scores. Mean CADESI-04 scores improved significantly one month after treatment and remained stable until the last follow-up. Data represent mean±sd; **P<0.001 versus baseline. CADESI-04, Canine Atopic Dermatitis Extent and Severity Index, version 4.

In vitro multilineage cell differentiation

cAd-MSCs differentiated into all three target phenotypes when cultured in the presence of appropriate induction medium. Adipogenic differentiation was confirmed by Oil Red O staining, present in the cytoplasm red lipid droplets. Under osteogenic conditions, cells formed white nodule-like aggregations, which were strongly stained for alkaline phosphatase activity and Alizarin Red S. For chondrogenic differentiation, 3D pellet exhibited metachromasia when stained with toluidine blue, indicating a cartilaginous matrix.

Inhibition of proliferation of allogeneic PBMCs

Statistically significant (P<0.05) cAd-MSC suppression in the proliferation of allogeneic PBMCs when stimulated with ConA was found in mixed cell cultures compared with control values (figure 2).

Karyotype

cAd-MSCs had a normal metaphase spread and karyotype (figure 3).

Treatment outcome

Twenty-six dogs were initially included in this study (table 2). Four dogs were excluded during the follow-up because they needed immunosuppressant medication to counteract inflammation and pruritus. One of them was withdrawn before the three-month appointment and the other three before the six-month follow-up. These four dogs were not included in the statistical analysis.

Initial CADESI-04 and owner pruritus VAS scores for the animals that completed the study were (mean±sd (range)) 35.4±21.4 (12–84) and 8.36±1.1 (6–10), respectively. The mean pruritus VAS score improved significantly (P<0.05) from week 1 (6±2.28, (3–10)). The mean CADESI-04 score improved significantly (P<0.001) after the first month (12.09±12.07 (1–65)). This recovery remained stable until the last follow-up and did not show signs of regression or worsening.

Six months after treatment, CADESI and pruritus VAS showed basal levels, 9.73±17.02 (0–82) and 1.86±2.25 (0–8), respectively (figures 4 and 5). Owner's global assessment of treatment efficacy score was 2.15±1.15 (0–3) over 3 for all animals that started the study.

Neither local or systemic adverse reactions were observed in relation to intravenous administration of allogeneic cAd-MSCs. All haematological and serum chemistry parameters did not show relevant changes within the initial reference ranges on all study time points.

Discussion

This study evaluates clinical and safety results after systemic implantation of allogeneic cAd-MSCs in a major animal model with natural AD refractory to conventional treatment. The clinical results of this study show CADESI-04 scores improved significantly after the first month post-treatment, and remained stable until the last follow-up, at six months, without signs of regression or worsening. Mean owner pruritus VAS scores showed similar evolution and improved significantly from one week after treatment. Owner's global assessment of treatment efficacy score was 2.15±1.15 (mean±sd) over 3. Although this assessment is a non-validated scoring system and any conclusion should be taken with caution, it is important to highlight that the group of animals had an AD history of 2.54±1 years.

The results of the present study, despite the possibility of being conditioned by bias due to the uncontrolled nature of the study, have shown statistically significant changes in some measured parameters. It is important to heed that patients were refractory to conventional therapy and that immunomodulatory medication was suppressed two weeks before the study.

Of the 26 refractory AD treatment animals, 22 finished the study without systemic immunosuppressant medication at six-month follow-up. None of the animals showed systemic or local adverse events after allogeneic cAd-MSC administration. This is in accordance with other MSC therapies described in canine species.[19 20 23 35] Four dogs (15 per cent) did not respond to the cell therapy and needed additional therapies not compatible with continuation in the study. As a result, score evolution data were not included in the statistical analysis. This may have created bias due to the small sample size population. It should be noted that three of them (12 per cent) did not need immunosuppressant medication for the first three months of follow-up. Therefore, it would be interesting to study the results of multiple administration of cell therapy or those associated with medication.

The authors' cAd-MSCs meet the minimal requirements of core identity, purity and immunomodulatory potential required by the International Society for Cellular Therapy.[36 37] The authors evaluated the inhibitory capacity of cAd-MSCs in the proliferation of allogeneic PBMCs despite mitogen stimulation. This capacity has been shown similar to other domestic species and human MSCs.[16 38 39] Their genetic stability was also demonstrated by means of karyotyping.

Different in vivo studies evidenced that systemically infused MSCs homing to injured and inflamed tissues resulted in a positive therapeutic effect.[28 40–42] In AD, MSCs migrate to skin lesions through draining lymph nodes[28] and decrease physiological migration of interstitial dendritic cells from the skin to lymph nodes. At the same time, MSCs decrease cell infiltration in the skin lesion, reducing generation of effector T cells.[28] Dendritic cells are the most potent type of antigen-presenting cells and play crucial roles in the initiation and control of the adaptive immune responses.[43] In addition, some MSCs may remain in secondary lymphoid organs to block the migration of effector T cells to the inflamed region.[28 43]

As far as the authors know, only one previous autologous MSC cell therapy study in canine AD has been published,[44] having important limitations such as a small number of patients (five), with only three of them completing the follow-up period (three months), and the limited improvement in clinical signs.

Recently, human MSCs derived from different tissues affected by AD showed immunomodulatory profile differences compared with healthy subjects, evidencing that MSCs could be involved in the AD pathogenesis,[45] as has been described in other immune-mediated diseases.[46–48] This would support the idea of an allogeneic MSC therapy in AD.[27]

Considering that dogs develop AD naturally with cutaneous clinical characteristics similar to human beings, despite some differences,[5 10 49 50] this study might serve as a partial model for human AD therapy with cell-based regenerative strategies.

The current work is an experimental study with promising results suggesting the efficacy of allogenic MSCs for the treatment of AD in dogs. This treatment is available for research purposes only. Currently there is no commercial option available, and an estimation of the cost of this kind of treatment will be strongly influenced by the requirements of the regulatory agencies.

The authors are aware that the design of this study has important limitations: fundamentally open-label, limited number of patients and lack of control group, which could affect the evaluation of the results. To minimise the effect of seasonality on the symptomatology of the patients, all cellular therapies were administered within a six-week period.

Bearing in mind these limitations and being the first pilot study of allogeneic cAd-MSC therapy in refractory AD, the results of this study were associated with an improvement in clinical score (CADESI-04) and pruritus for at least six months. Furthermore, it has been shown that both dose and route of administration do not produce adverse events, which is in accordance with other results published by the present authors for different pathologies and domestic species treated with MSCs.[19 20 51]

Conclusion

In this study, a single systemic administration of allogenic cAd-MSCs appeared to produce positive results in the remission of clinical signs of canine refractory AD, for at least six months and without adverse events.

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Competing interests None declared.

Ethics approval All animal procedures and protocols were conducted by licensed veterinary surgeons and comply with both national and European legislation (Spanish Royal Decree RD1201/2005 and EU Directive 86/609/CEE, as modified by 2003/65/CE, respectively) for the protection of animals used for research and experimentation and for other scientific purposes. Likewise, the Institutional Animal Care and Use Committee, Andalusian Centre, approved the protocols for Nanomedicine and Biotechnology (BIONAND), Málaga, Spain.

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