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Effect of allogeneic mesenchymal stem cells (MSCs) on corneal wound healing in dogs

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1. Introduction

The cornea of small animals is composed of five layers: pre-corneous lacrimal film, epithelium, stroma, descemet's membrane and endothelium¹ being able to transmit light due to the avascularization of the epithelium, absence of pigments, regular arrangement of collagen in the stroma and maintenance of hydration.² The peculiar organization of the collagen, together with the absence of blood vessels, maintains cornea transparency to allow the passage of light,³ and the integrity of the cornea plays a

critical role in maintaining visual function due to its transparency.⁴ Corneal ulceration is one of the most common conditions in veterinary ophthalmologic clinics, and may be caused by trauma, infection, inflammation, among other factors. This occurs when the epithelium and part of the stroma layer is lost.⁵ This mainly causes loss of transparency of the initial cornea, especially in cases where adequate therapy is not applied, with progression to ocular perforation, presenting possibly irreversible lesions and consequent loss of vision.⁶

The lesions are classified according to its depth, and in superficial ulcer, lesions cause corneal epithelial loss,^{2,7} ocular discomfort with intense photophobia, blepharospasms, increased tear production, conjunctival hyperemia and corneal edema.⁵ In the case of deep ulcers, lesions involve more than half of the corneal thickness, affecting the stromal layer, with signs of inflammation, corneal edema, pain, purulent secretion, signs of anterior uveitis.² Currently, the form of treatment is related to the degree of the lesion.

The therapeutic conducts are based on the relief of the clinical signs and symptoms, prophylaxis or in the control of infection, erosion and withdrawal of the cause. When the ulcer is superficial, the therapeutic course may be clinical, but in deep ulcers and in perforation cases, the therapeutic course includes surgical procedures associated with drug therapy.^{2,7,8} The treatment of choice is usually surgical intervention⁹; however, recent studies have shown the beneficial effects of stem cell-based therapies on ocular surface healing, specially using mesenchymal stem cells (MSCs) or its secreted factors.¹⁰

The effects observed using MSCs, when transplanted into an organism, occur mainly due to the release of bioactive molecules (adhesion, extracellular matrix proteins, cytokines, growth factor receptors) that allow an interaction with the environment in which

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List of abbreviations

MSCs	mesenchymal stem cells
MHC	major histocompatibility complex
PBS	phosphate-buffered saline
MPO	myeloperoxidase
Disc	discomfort
CD	cluster of differentiation
SOX-2	sex determining region Y-box 2
OCT 3/4	octamer-binding transcription factor 3/4

they are transplanted.¹¹ Interestingly, when fused to the cells of the organism they are able to modulate the inflammatory response and mitosis of the cells and this promotes tissue repair and immunomodulation^{12,13,14}

Thus, this study has as main aim to evaluate the use of MSCs in dogs with corneal wounds. We believe that the results presented here are important in order to improve post-operative recovery, correct unsatisfactory results, accelerate and promote corneal recovery and intensify the uses of therapy with MSCs, which are the most promising source of cell proliferation and differentiation.¹⁵

2. Materials and methods

2.1. Isolation, culture and freezing of MSCs

MSCs were isolated and cultured from adipose tissue of one health dog donor, according to the previously described protocols^{16,17,18}. For the collection of adipose tissue, the donor was anesthetized, an incision was made in the lumbar region, and approximately 20 g of adipose tissue was collected from the base of the tail. The adipose tissue was washed in saline phosphate solution to remove cellular and blood residues, cut in small pieces and then, exposed to hyaluronidase, to go through an enzymatic digestion. After this, the cells underwent a filtration process in order to initiate a selection of the MSCs. Afterwards, the cells were placed in culture bottles with Dulbecco's modified Eagle's medium (DMEM), which were incubated at 37.5 °C and 5% CO₂. After 24 h, the medium was discarded, with the non-adherent cells, and fresh culture medium was added to the bottles. The medium was changed once every 3 days, until cells reached 80% confluence, when trypsinization was performed to take the cells off the bottles, count in Neubauer chamber and pack them in straw (1 million cells/straw) for freezing with DMSO and BFS in liquid nitrogen, as previously described.^{19,20} Five straws (5 million cells) were thawed for characterization of MSCs.

2.2. Characterization of MSCs

MSC characterization tests were performed as determined by the International Society for Cellular Therapy.²¹ The surface markers positive for the MSCs tested were mouse anti-human CD29-RD1, mouse anti-equine CD44-FITC, caprine anti-canine CD90 primary and mouse IgM anti-caprine conjugated AF594 (secondary). The negative surface marker was mouse anti-human CD34-FITC. The capacity to differentiate of MSCs was also analyzed by the presence of two transcription factors for cell differentiation: SOX2 and OCT3/4. These markers were evaluated by the immunophenotyping method on Amnis® Imaging Flow Cytometer.

The ability of the cells to differentiate into osteoblasts, adipocytes, and chondrocytes was also evaluated, as previously

described.^{18,22} The culture medium of the cells were screened for pathogens and contaminants (e.g., bacteria, fungi, mycoplasma), using polymerase chain reaction (Veriti Thermal Cycler – ThermoFischer Scientific®). Also, viability of the cells after thawed was evaluated by flow cytometer with Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (PI) (ThermoFischer Scientific®) on Amnis® Imaging Flow Cytometer.

2.3. Animals

Eligible subjects were dogs who were diagnosed with deep corneal ulcers by an ophthalmology clinician based on previously established criteria.²³ The twenty-six (26) selected dogs had been previously treated for 15 days with allopathic drops (antibiotic and tear repository) and presented no corneal wound healing. The animals did not receive any kind of immunomodulatory medication for at least 2 weeks before and for the entire duration of the study. All dogs owners signed a written consent before initiation of this experimental procedure and were fully informed that safety, complications, and efficacy of the cell implantation in corneal ulcer were not known.

Ophthalmic examination was performed three times: prior to MSC therapy, 7 and 14 days after therapy. The analyzes included criteria such as the discomfort of the animal due to the ulcer and the depth of it. The discomfort of the subject was (0) absent, (–1) mild, (–2) moderate, and (–3) intense. The depth of corneal ulcer was evaluated based on Kern (1990): (1) superficial, (2) anterior stroma, (3) posterior stroma (4) descemetocoele, (4+) corneal melting, (5) indolent.

Animals with diagnosed comorbidities or those with superficial corneal ulcer at baseline were excluded from the clinical study.

2.4. Preparation and application of allogeneic mesenchymal stem cells (MSCs)

The laboratory produced and conserved the MSCs in frozen straws. MSCs were thawed and washed for use, according to our protocol, using specific washing and transport medium. For each treatment, 3 million MSCs were used, and the application was performed as follows: an initial application of 0,25 ml of transport medium containing 250,000 cells by sub-conjunctival route performed by a veterinarian ophthalmologist followed by 11 instillations (one per hour) of 0,25 ml of transport medium containing 250,000 cells. Each animal returned for ophthalmologic evaluation at 7 and 14 days after treatment for a case follow-up. Considering corneal ulcer in domestic animals is a traumatic lesion that can occur at any time, no other follow-up was performed after 14 days.

From the time of diagnosis of the corneal ulcer to the period of 14 days after therapy with the MSCs, the animals received ocular instillation of antibiotic eye drops and tears repository to control possible infections and support the action of cells that were applied.

2.5. Adverse and side effects

During the clinical study, the analysis of adverse and side effects was carried out, including the evaluation of the occurrence of any intercurrent during and until 14 days after the therapy with the allogeneic MSCs.

2.6. Statistical analysis

The data were transformed into numerical variables, which were submitted to statistical analysis for differences between pairs

ordered by the Wilcoxon non-parametric test using the SAS Program (Cary, North Carolina, v.5.1) (see Fig. 1).

3. Results and discussion

MSCs from the donors were properly isolated and characterized by our laboratory. After 24 h of isolation, small colonies of adherent cells were scattered across the bottom of the bottles (Fig. 1A). After

2 days, the cells began to grow and elongate, becoming a spindle shape similar to fibroblasts. When the cells reached 80% of confluency, they had typical phenotypic characteristics of MSCs, and were attached to the bottom of the bottle with a spindle-like shape and self-renewal ability (Fig. 1B). The flow cytometry results after the cells were thawed, demonstrated that MSCs expressed high levels of CD29 (Fig. 2A), CD44 (Fig. 2B) and CD90 (Fig. 2C) but did not express the hematopoietic marker CD34. There was also a high

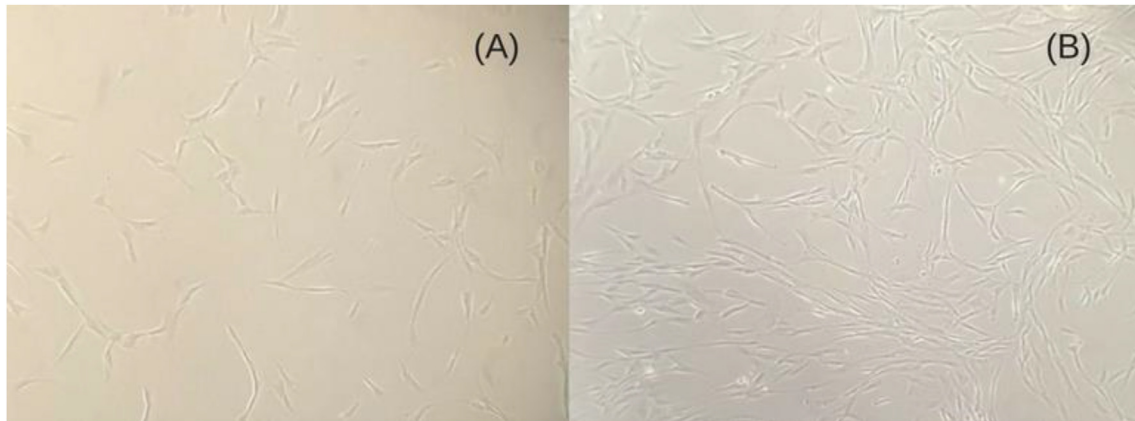


Fig. 1. Adipose tissue-derived Mesenchymal Stem Cells (MSCs) under microscopy. (A) Small colonies of adherent cells scattered across the bottom of the bottles; (B) Cells attached to the bottom of the cell culture flask exhibiting a spindle shape.

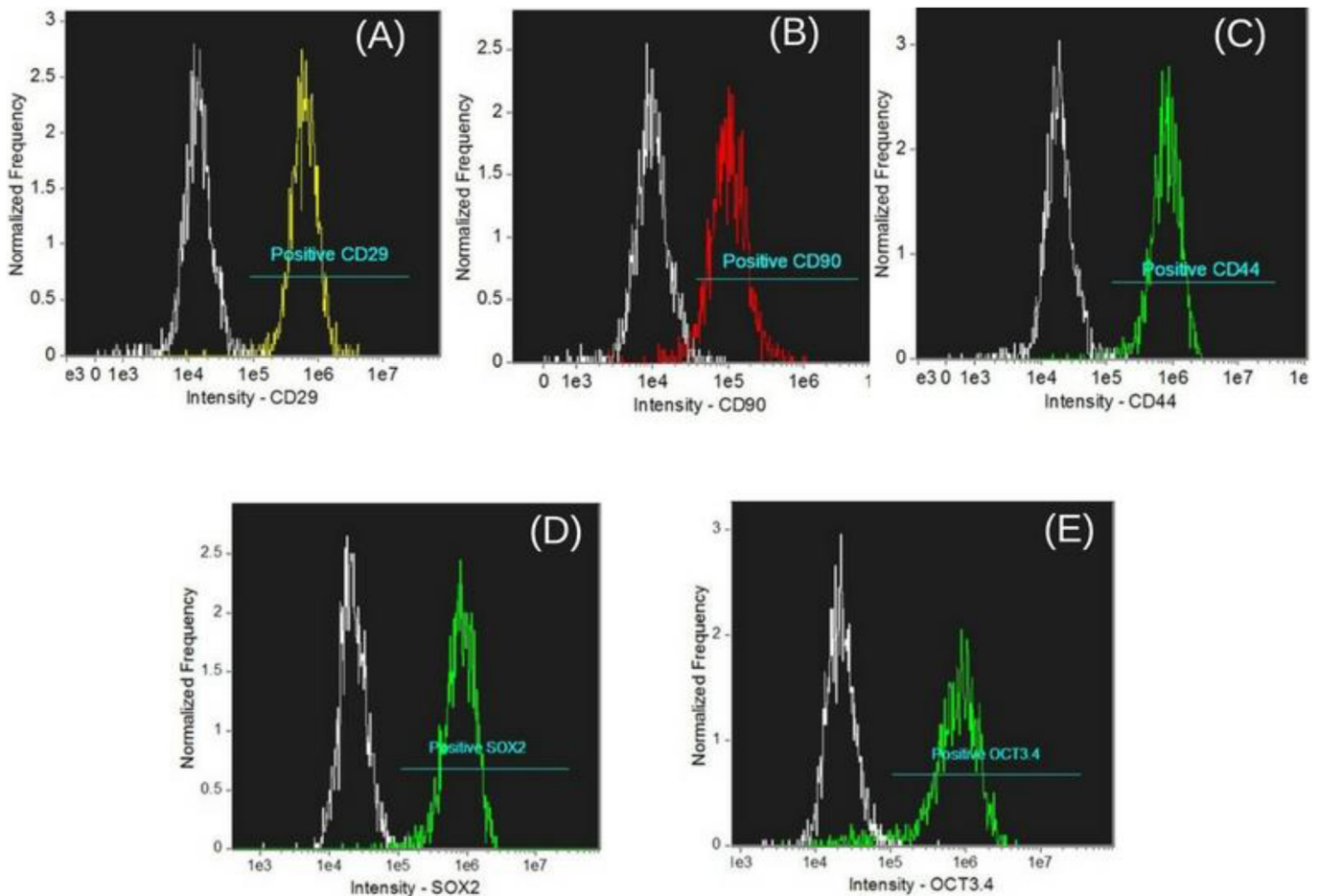


Fig. 2. Flow cytometry results, with representative immunophenotype profiles of canine MSCs for several MSCs markers. Flow cytometry analysis revealed a homogeneous cell population, characterized by the positive expression of CD29 (A), CD90 (B) and CD44 (C). Cells also express intracytoplasmic markers SOX2 (D) and OCT3/4 (E).

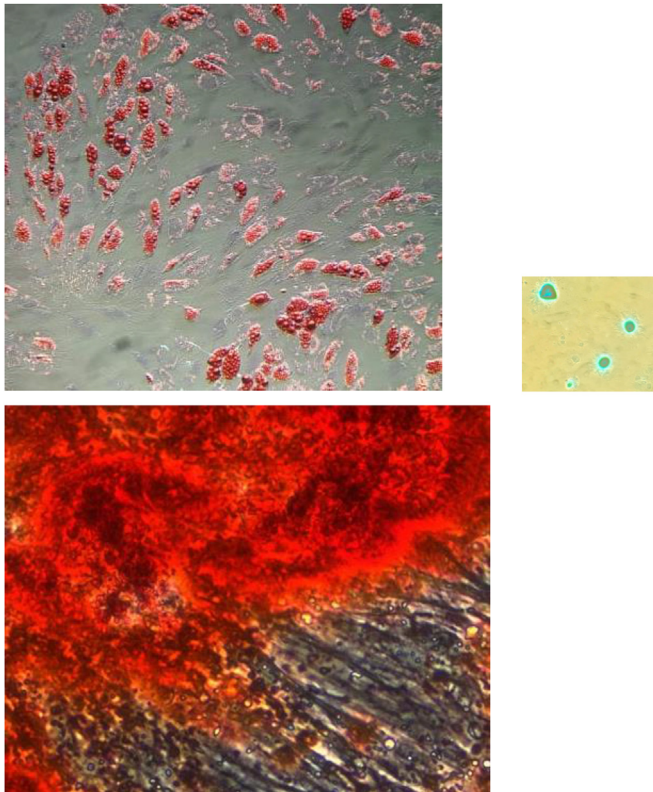


Fig. 3. Mesenchymal stem cells induced to differentiation into adipogenic (A), chondrogenic (B) and osteogenic (C) lineages.

expression of SOX-2 (Fig. 2D) and OCT3/4 (Fig. 2E). The analysis of alive, necrotic, apoptotic and dead cells using the flow cytometer, showed a percentage of 80.6% viable cells 2 h after thawed (data not shown). These results express that MSCs obtained from the donor have shown consistency in their isolation, expansion, high ratio proliferation, plastic adherent, and behavior *in vitro*, exhibiting its ability of adipogenic, osteogenic, and chondrogenic differentiation, similar to those previously described (Fig. 3).²²

MSCs are multipotent, self-renewing and can facilitate wound repair by modulating cellular responses in the cells of surrounding tissue and immune cells, creating a pro-regenerative and anti-inflammatory environment.²⁴ These features can be useful in the therapy of depth corneal wounds, which are common types of ocular injury in small animals, and the current procedure to treat it is the surgery. The corneal epithelium is self-renewing, and this process is essential for normal vision, but in cases of depth wounds, other layers are affected besides the epithelium.⁵ The subconjunctival route was chosen due to its capability of releasing higher levels of the medication to the eye for an extended period of time²⁵ and, was accompanied by topical instillation, so a higher quantity of cells could be delivered in a short period of time – 12 h and survive for 3–5 weeks,²⁶ enough time to promote the wound healing.

The cohort of 26 dogs selected for this study were all refractory to 15 days previous treatment (antibiotics and tear repository drops), in other words, none of them had natural healing of the ulcers and received the therapy with MSCs. After allogenic MSCs therapy, 22 out of 26 dogs presented complete ulcer wound healing within 14 days, totaling 84.6%. One animal did not go through the 7 and 14 days evaluations due to the severity of the case and it was submitted to surgery 4 days after the cellular therapy (Table 1). Other 3 animals had no improvement of corneal ulcer and were latter submitted to surgical procedure and it is unknown why they proved unresponsive. However, the animals were treated at home

Table 1
Effect of allogenic MSCs transplantation on corneal healing and degree of discomfort.

Animal Number	Group of Animals treated with MSCs					
	Before Therapy with MSCs		7 days after therapy with MSCs		14 days after therapy with MSCs	
	Depth	Disc	Depth	Disc	Complete Healing	
1	3	-2	0	0	Yes	
2	3	-3	1	-1	Yes	
3	5	-1	1	0	Yes	
4	3	-2	0	0	Yes	
5	3	-3	0	0	Yes	
6	5	-2	2	-1	Yes	
7	4+	-3	1	-2	Yes	
8	3	-1	1	0	Yes	
9	4	-3	1	0	Yes	
10	4+	-3	1	-1	Yes	
11	4	-1	1	0	Yes	
12	4+	-3	1	-1	Yes	
13	4	-2	1	0	Yes	
14	4	-2	1	0	Yes	
15	3	-2	0	0	Yes	
16	3	-2	1	0	Yes	
17	4	-2	1	0	Yes	
18	4+	-3	3	-2	No	
19	4	-2	1	-1	Yes	
20	3	-2	1	0	Yes	
21	4	-2	1	0	Yes	
22	4	-3	3	-1	No	
23	3	-2	1	0	Yes	
24	4	-2	1	-1	Yes	
25	4+	-3	-	-	No	
26	4+	-3	4+	-3	No	

*Depth (1) superficial, (2) anterior stroma, (3) posterior stroma (4) descemetocoele, (4+) melting corneum, (5) indolent; Disc: discomfort (0) absent, (-1) mild, (-2) moderate, and (-3) intense.

by the tutors after the cell therapy, attending to the ophthalmological evaluations only in the defined days. Next studies should take it into consideration to control this aspect.

The degree of depth (Fig. 4) significantly decreased during 14 days follow-up period ($p < 0.0001$) (Fig. 5).

This non-invasive method (cell therapy), appears to be a simple solution to substitute the surgery, with satisfying results. According to previous studies, MSCs promoted reduction of inflammation, mobilization of endogenous stem cells and increased concentration of growth factors when treating corneal damages^{4,27,28} stimulating a rapid recovery, as occurred in a large part of the study animals.

Several studies have been previously performed treating corneal wounds with MSCs in rats and rabbits, reporting an improvement of the corneal surface, probably due to the secretion of cytokines, responsible for the increase in anti-inflammatory response and mobilization of endogenous MSCs to the injured area.^{28–30} A study with mice induced to type 1 diabetes and consequent chronic corneal defects showed markedly decreased inflammatory infiltrates in the histology of these diabetics' corneas accompanied by lower ELISA levels analyses of MPO (a biomarker of activated neutrophils and inflammatory cytokines) when compared to corneas treated with placebo.³¹

In accordance to our results, Bermudez et al. (2015) showed that even the medium of culture of MSCs can be used to enhance wound healing and reconstitute corneal surface of chemical burned mice eyes, demonstrating that the main effect must be due to the cytokines secreted by the cells. This study showed the improved epithelial regeneration and reduced corneal macrophage inflammatory protein-1 alpha (MIP-1a) and TNF-A mRNA expression. It was also suggested that this medium contain a bactericidal effect, due to the elevated levels of some chemokine (CX-C motif) ligands 1, 6, 8, 10 in it. These are promising results to the veterinary ophthalmology, considering the possibility of development of a non-invasive method to treat depth corneal ulcers.

Other studies have demonstrated the *in vitro* ability of MSCs to form stratified epithelial tissue with characteristics and expression of epithelial cell markers,³³ suggesting that the damaged epithelium in cases of corneal wound, is remade with the presence of MSCs and the growth factors released by them. Yao et al. (2012)



Fig. 4. Images of the left eye of dog number 7 with melting deep ulcer at baseline (depth 4+) (A) and 14 days after cell implantation (B). Left eye of dog number 17 (depth 4) at baseline (C) and 14 days after cell implantation (D).

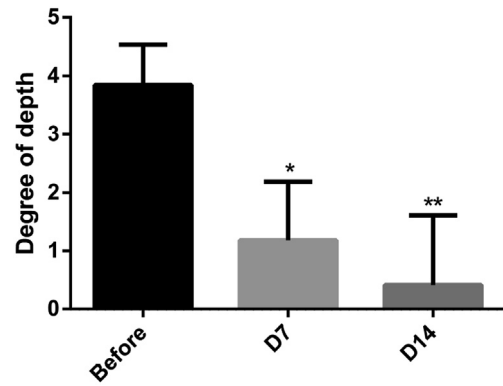


Fig. 5. Degree of depth of the ulcer before and after the treatment of the dog's eyes. Mean and Standard Deviation (SD) of the degree of depth of the corneal lesion before and 7 days after the application of stem cells. The asterisk indicates significant difference between the columns ($p < 0.0001$).

tested subconjunctival injection of MSC in rats with corneal damage and concluded that the recovery of corneal surface (analyzed by staining with fluorescein) was significantly faster in animals that received MSCs when compared to animals that received phosphate-buffered saline (PBS). These results, in accordance to ours, suggest that MSCs have an anti-inflammatory effect explaining its efficacy.

The degree of discomfort of the animals with the ocular lesion before the therapy was mild (2 cases), moderate (13 cases) and intense (10 cases), present to absent (15 cases), mild (7 cases) and remained intense in 1 case 7 days after MSC therapy. For this variable, there was a significant difference between the evaluations performed before and after treatment and on day 7 ($p < 0.0001$) (Fig. 6). This is an important result, since it is a clinical response strictly related to the animal welfare, a big concern of tutors and veterinarians. In addition, the degree of discomfort is directly related to the possibility of new lesions occurring, since an uncomfortable animal is more likely to manipulate the injured eye in order to relieve discomfort.

The limitation of our study was the impossibility to use control animals. Due to the clinical precedence of the dogs, there was no possibility of leaving animals without treatment or receiving only placebo. Also, the animals selected to the study have been previously treated only with antibiotics and tears repository, to control the possibility of self-healing, which did not happen in any of them.

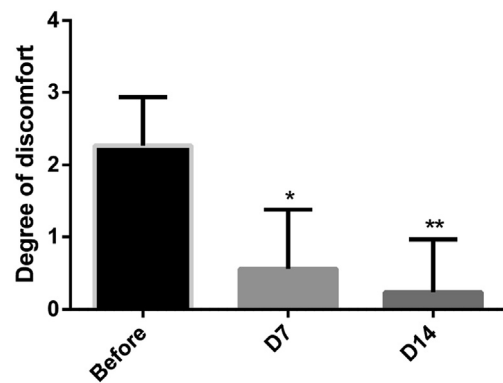


Fig. 6. Degree of discomfort before and after the treatment of the dog's eyes. Mean and Standard Deviation (SD) of the degree of discomfort before and 7 days after the application of stem cells (D7). The asterisk indicates a significant difference between the columns ($p < 0.0001$).

The final data from our clinical study showed that none of the animals had any adverse reaction or side effects due to the application of allogeneic MSCs, indicating that the cells were well tolerated by the individuals, as previously suggested by studies that have identified that this type of treatment is safe because of low MHC I expression and the absence of MHC II in canine adipose derived MSCs.³² Several studies, including clinical trials, have shown similar results, presenting the safety of allogeneic stem cell therapy^{34,35,36}.

4. Final considerations & perspectives

The present study demonstrated that allogeneic MSC therapy is effective in promoting the healing of corneal wounds in varying degrees of depth in dogs. The result of 84.6% of the cases reversed without the need of any surgical intervention, significantly reducing the degree of depth of the wounds is very encouraging. We could also conclude that the use of allogeneic MSCs was a safe, effective and fast-acting form of treatment of deep corneal wound in small domestic animals. We believe that, despite this study being carried out in dogs, it can be useful in the development of treatments to human eye healing conditions, like corneal burn or any corneal epithelium loss. Since the dogs studied were from a varied background, with different conditions and depth of corneal wounds, the effectiveness of treatment and our results are of significance. Finally, we believe that new studies could be performed in other animal models (cats, equines) to verify the similarities between species. From our knowledge, this is the first study using allogeneic MSCs to treat corneal wounds in dogs.

Conflicts of interest

The authors declare no conflicts of interest in this work.

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