SUBCONJUNCTIVAL USE OF MESENCHYMAL STEM CELLS FOR THE TREATMENT OF CANINE ULCERATIVE KERATITIS

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Abstract. Ophthalmic diseases are common problems in dogs of various breeds and ages. In recent years, ophthalmologists have been paying more attention to stem cell (SC) therapies, since the renewal and regeneration of any tissue in the adult body depends on somatic SC, and eye tissues are no exception. The aim of the present work was to determine the influence of allogenic mesenchymal stem cells (MSC) in healing ulcerative keratitis of dogs. Our research showed that subconjunctival injections of allogeneic MSCs from adipose tissue was clinically safe for use in dogs during the follow-up period. These injections contributed to the decrease of the clinical manifestations of ulcerative keratitis in dogs, as evidenced by a decrease the intensity and area of the affected areas of the cornea compared to classic therapy.

Keywords: ophthalmic diseases, ulcerative keratitis, allogeneic mesenchymal stem cells, MSC transplantation, subconjunctival injections, cell therapy.

Introduction

Ophthalmic diseases are observed in most animals of various breeds and ages. Preserving an animal's vision is one of the most important tasks for preserving their quality of life. Without an exact diagnosis, eye treatment is rarely successful. Unfortunately, owners cannot always recognize the first signs of ocular disease, they may no contact a veterinarian promptly, or, upon noticing any symptoms they may try to treat the animal themselves (Mandell & Atkins, 2022). If corneal damage does not receive relevant treatment vision will be lost (Hendrix, 2014). Different breeds of dog have various predispositions for ulcerative keratitis. Pugs (5.42%), Boxers (4.98%), Shih Tzus (3.45%) are breeds which represent the highest prevalence of ulcerative keratitis (O'Neill *et al.*, 2017). According to the literature dogs with nasal folds are nearly five times more likely to be affected by ulcerative keratitis than those without. Brachycephalic dogs (craniofacial ratio <0.5) are twenty times more likely to be affected than non-brachycephalic dogs. Research has shown that s 10% increase in relative eyelid aperture width more than triples the risk of ulcers (Packer *et al.*, 2015).

Ulcerative keratitis frequently heals spontaneously because the corneal epithelium maintains a high level of proliferative activity. Alongside this keratectomy may prove useful in the early stages of ulcerative keratitis. This method leads to minimized scarring and decreases the stimulus for keratitis and iridocyclitis (Grahn *et al.*, 2004). However, sometimes, ulcer healing capacities are diminished, even when standard treatments were administered Bremond-Gignac *et al.*, 2019). Therefore, a few studies conducted in the last few years have evaluated the efficacies of different therapeutic methods for ulcerative keratitis, including the use of platelet-rich plasma (Farghali *et al.*, 2021), bovine freeze-dried amniotic membrane[8], and administration of other drugs (Bremond-Gignac *et al.*, 2019; Martinez *et al.*, 2019). In recent years, ophthalmologists have been paying more attention to stem cell (SC) therapies, since the renewal and regeneration of any tissue in the adult body depends on somatic SC, and eye tissues are no exception (Joe & Gregory-Evans, 2010; Oner *et al.*, 2018). There is data available showing that SCs have a high potential in relation to the treatment of eye diseases characterized by irreversible cell loss,

such as glaucoma, age-related macular degeneration, photoreceptor cell degeneration, hereditary retinopathy, mechanical and ischemic retinal lesions (Song *et al.*, 2015; Zarbin *et al.*, 2016). Despite this, subconjunctival injection of SC in the perilimbal region have not indicated any clinical improvement in canine patients with chronic superficial keratitis. It is possible that SC introduced into localized areas cannot exert an influence on this autoimmune disease (Pereira *et al.*, 2022).

The aim of the present work was to determine the influence of allogenic mesenchymal stem cells (MSC) in healing ulcerative keratitis of dogs.

Materials and Methods

The research was performed at the Faculty of Surgery, Obstetrics and Pathology of Small Animals, at the Kazan State Academy of Veterinary Medicine and Kazan Federal University. Animals were selected, and their feeding and environmental conditions were identical. Three groups were identified: One control group and two experimental groups, each containing 4 animals. The ages of the animals ranged from 6 months to 12 months, and each dog weighed 2-4 kg. Previously, these animals had been diagnosed with ulcerative keratitis by a veterinarian. This diagnosis was accompanied by general depression, decreased appetite, normal or slightly elevated body temperature, pronounced blepharospasm, edema of the eyelids, conjunctiva, sensitivity, and the presence of a crater-like defect on the cornea. The diagnosis was made following visual examination, ophthalmoscopy (ophthalmoscope Eurolight KaWe E36, Russia), and examination of the anterior segment of the eye with a slit lamp Monvet-4 (Russia).

To identify the frame and depth of corneal defects, it was stained with 1% fluorescein solution (Apicenna, Russia). The depth and diameter of the corneal defect at the entrance and in dynamic movement were determined using a caliper. The etiology of this disease in animals within the experimental and control groups was naturally occurring mechanical damage of the eyes due to trauma.

Surgery

The third eyelid flap technique was performed as follows. The animals were fixed in a lateral position after anesthesia, so that the affected eye was on the upper surface. The surgical field was prepared and a traditional manner with the fur removed around the area and the dirt removed from the skin around the eye, thereafter it was degreased and disinfected with a 70% rectified alcohol solution. A q - tip soaked with a sterile 0.9% saline solution was used to remove the unviable epithelium from the corneal surface (Fig. 1a). The third eyelid was grasped with eye tweezers and fixed to the external corner of the eye with a loop-shaped suture, without affecting the T-shaped cartilage of the third eyelid (Fig. 1b, c). Furthermore, the animals were prescribed ambulatory treatments. Antibacterial Tobrex (ALCON-COUVREUR N.V., Belgium) eye drops were prescribed using 2 drops three times a day. A 30 min application of Corneregel (Dr. GERHARD MANN Chem.-Pharm. Fabrik, Germany) was used locally twice a day to stimulate regenerative processes in the cornea. After 14 days, the suture was removed from the eyelids, the condition of the cornea was assessed. Gilan (Grotex, Russia) eye drops were then prescribed to moisten the cornea twice a day for 14 days. Examinations of each animal and checks on corneal defect healing were performed once every 7 days for 4 weeks (Shivraj *et al.*, 2011).

Isolation of mesenchymal stem cell

A culture of mesenchymal stem cells was isolated from the visceral fat of a dog after a planned ovariohysterectomy. All of the cells were isolated and characterized according to the standard procedure described earlier (Zakirova et al., 2021). The cells were analyzed for MSC membrane markers using a flow cytofluorimeter FACS Aria III (BD Biosciences, USA) after aliquot staining using conjugated antibodies according to the manufacturer's instructions (Table 1). For treatment concentration of MSCs was $1.25*10^6$/ml.

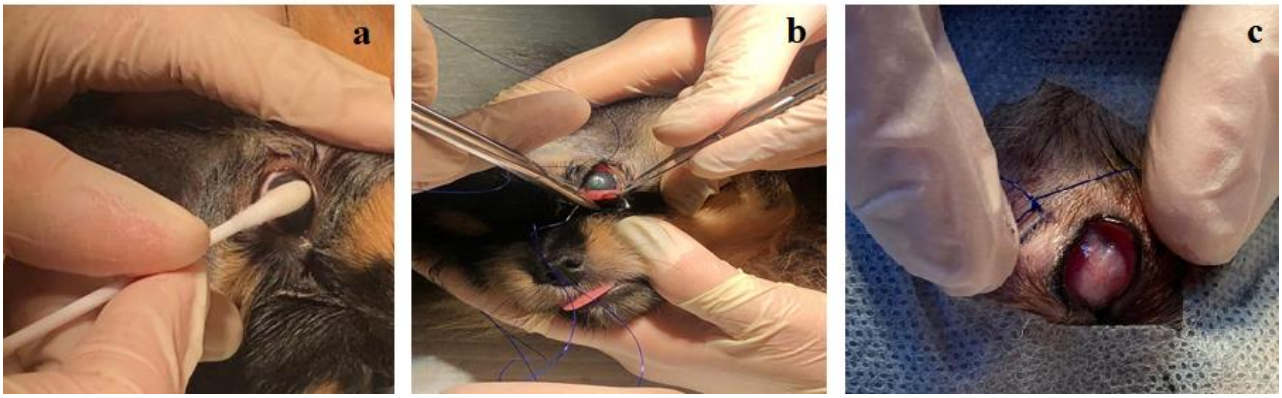

Fig. 1. Photographs showing the tarsorrhaphy technique. a) corneal surface preparation prior to tarsorrhaphy; b and c) execution of tarsorrhaphy

Table 1

List antibodies for MSC characterization

Marker Name	Monoclonal antibodies			
	Clone	Species reactivity	Species	Manufacturer
Thy-1 (CD90), conjugated with PE/Cy5	5E10	Human, African green monkey, baboon, rhesus macaque, pig	Mouse	Biolegend, USA
CD166 conjugated with PE	3A6	Human	Mouse	Sony, USA
CD44, conjugated with PE	IM7	Mouse, human, baboon, chimpanzee, cynomolgus, rhesus macaque, squirrel, monkey, horse, cattle, pig, dog, cat	Rat	Biolegend, USA
CD73, conjugated with PE	AD2	Human, African green monkey, baboon	Mouse	Biolegend, USA
CD29, conjugated with PE	HMβ1-1	Mouse, rat	Armenian hamster	Biolegend, USA

Experimental groups

For group I (control), the treatment of the animals consisted of third eyelid flap technique, after combined anesthesia, for a period of 14 days. 1% Meditin (Apicenna, Russia), 30 mg/kg, was used as a sedative, and the general anesthesia was 1% Propofol kabi (FRESENIUS KABI DEUTSCHLAND, Germany) at 4 mg/kg, and the local anesthesia was a 0.4% solution Inocaine (Sentiss Pharma PVT. LTD, India) for superficial instillation of the cornea.

Treatment in the experimental groups (groups II and III) consisted of two stages. The group II animals received third eyelid flap technique, with the techniques and anesthesia described above. The animals were also prescribed ambulatory treatments in the form of antibacterial Tobrex eye drops, two drops three times a day and Corneregel was applied locally twice a day to stimulate regenerative processes in the cornea. After 14 days, the suture was removed from the eyelid, and corneal condition was assessed.

The second stage of treatment for group II consisted of three subconjunctival injections, applied at seven day intervals, of 25.6IU Lidase solution in a volume of 0.3 ml. Superficial instillation of the cornea was performed with a 0.4% solution of Inocaine providing local anesthetic. To moisten the cornea, Gilan eye drops were prescribed twice a day.

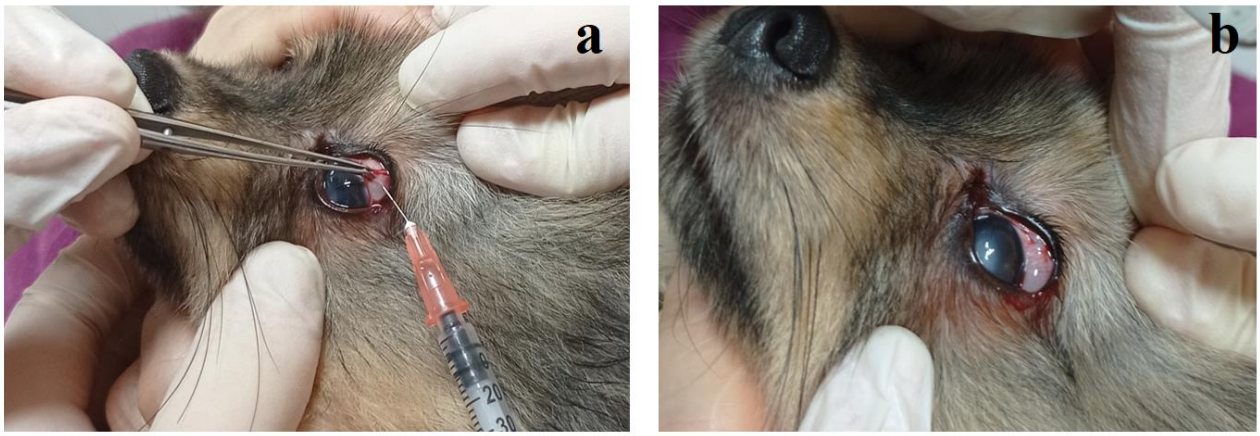

Fig. 2. Photographs showing the injection of suspended MSC in the conjunctival pouch. a) injection conjunctival pouch; b) eye after injection

The group III animals had third eyelid flap technique, as previously described, as their first stage of treatment. Thereafter they were proscribed ambulatory treatment using the antibacterial Tobrex eye drops, two drops three times a daily. Corneregel was used locally twice a day to stimulate regenerative processes in the cornea. After 14 days, the suture was removed from the eyelids, and the condition of the cornea was assessed. The second stage of treatment consisted from allogeneic MSC injection (concentration of 500,000 in 0.4 ml saline solution). MSCs were injected subconjunctively into the upper external quadrant of the affected eye, this process was applied three times, each injection had an interval of seven days between applications (Fig. 2). Superficial instillation of the cornea was then performed with a 0.4% solution of Inocaine used as a local anesthetic. Afterwards antibiotic therapy was performed for seven days in the form of localized Tobrex applications three times a day, and Gilan eye drops were prescribed two times a day to moisten the cornea. Corneal defect healing was monitored on the 14th day following suture removal, and on the 21st, 28th and 35th day of treatment.

Statistics

Data are presented as mean ± SD. Computer software (Excel 2016) was used to perform statistical analysis. Secondary statistical data processing was performed using the nonparametric Wilcoxon-Mann-Whitney U-test. A p value of less than 0.05 was considered to be statistically significant.

Ethics Approval

The protocol of this study was approved by the Biomedicine Ethic Expert Committee of Kazan Federal University (protocol 3; date 5 May 2015) under the institutional and international ethical guidelines. Injections and care were given in accordance with standard veterinary practice recommendations by qualified clinicians with additional health and welfare checks and clinical observations.

Results

The cells isolated from the canine adipose tissue adhered to the culture plastic and had a fibroblast-like morphology. Flow cytometry data are shown in Table 2.

The general condition of the animals at the start of the experimental was acceptable. Ulcerative keratitis was diagnosed via lateral examination (Fig. 3).

The surface area of the ulcers was rough, and the lower part was matte. Pain, photophobia, blepharospasm, hyperemia and edema of the conjunctiva and eyelids, abundant mucus, and corneal opacity were observed upon examination. The visual ability of the affected eye was classified as either reduced (50% of the animals), or absent (50%), depending on the diameter of the damage and the intensity of corneal

Table 2

Immunophenotyping conducted on the adipo-derived canine stem cells

Marker	Thy-1	CD44	CD29	CD73	CD 166
Number of cells expressing the marker in population, %	30	94	83	97	90

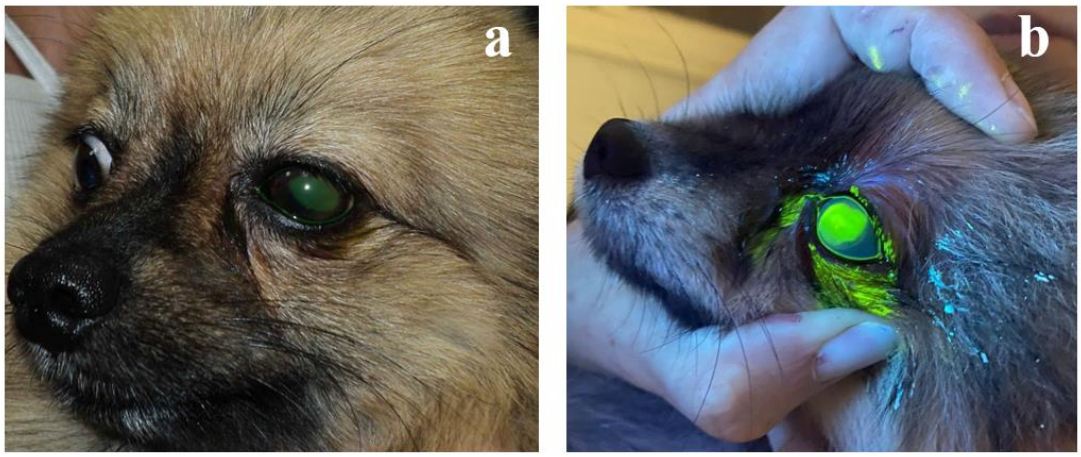

Fig. 3. Photographs showing the clinical diagnosis of the ulcer by fluorescent dye. a) eye suffered from corneal ulcer; b) after application of fluorescent dye showing the type and depth of corneal ulcers

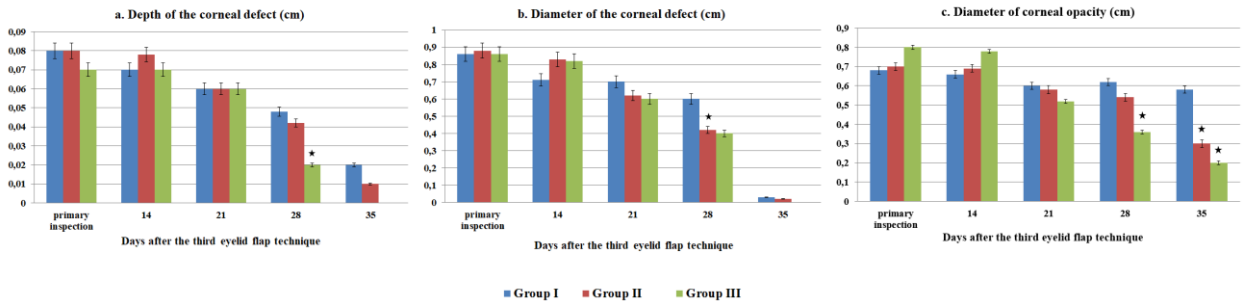

Fig. 4. Ophthalmological inspection outcomes of dogs
*$p < 0.05$ relative to the group I (control) (Wilcoxon-Mann-Whitney)

opacity. The depth and diameter of each defect within each dog showed approximately numbers in each of the three study groups (Fig. 4).

An appropriate general condition was observed in the animals after removal of the third eyelid flap technique (14d), and appetite was normal in all groups.

The localized clinical situation in the control group was characterized by using the following symptoms: lacrimal fluid secretion, deep corneal vascularization, scar on the cornea, and corneal opacity.

Upon inspection of the defect there was deep blood vessels sprouting into the cornea (vascularization) and the beginning of ulcer epithelization. Corneal opacity persisted. The results of the ophthalmological inspections are shown in Figure 4.

On the 35th day of treatment in group I (control), the general condition of the animals was acceptable and all of the animals had maintained their appetite. During a lateral inspection after fluorescein staining, the defect in the cornea was diagnosed. Partial epithelialization of the scar had occurred, and corneal opacification and deep corneal vascularization were ob-

served. The local clinical situation in the control group was characterized by the following symptoms: pain was absent, mucous secretion from the eyes was absent, deep vascularization of corneal was present, there was a decrease in the diameter of the scar on the cornea, scar epithelization and corneal opacity were present, and there was incomplete recovery of visual abilities. After treatment the visual ability had been preserved by 80% from health eye but corneal opacity persisted.

The general condition of the animals of group II was acceptable, appetite levels were maintained and the physiological indicators tested were within the normal range for this type of animal. After three subconjunctival injections of the Lidase solution the defect was diagnosed following fluorescein staining and a lateral observation. Scar epithelialization had occurred alongside corneal opacification and corneal vascularization. Inspection at the completion stage showed epithelialization in the cornea and visual ability had been preserved by 80% from health eye. Local corneal opacity was identified, the defect had become insignificant, and the scar was just 0.05 cm long.

The general condition of the animals in group III on the 35th day was also acceptable, appetite had been maintained and the physiological indicators did not exceed the normal characteristics for dogs. After threefold subconjunctival injections of MSCs, local expression of clinical signs of inflammation were not observed in any of the animals. The ocular defect was not diagnosed during the test with fluorescein, which indicates epithelialization of the ulcer. Local corneal opacification persisted, there was no scar at the defect. The visual ability of all of the dogs was measured at 100%. The localized clinical situation in this group was characterized by the following symptoms: local insignificant corneal opacity, corneal vascularization was absent, edema of the eyelids was not pronounced, corneal transparency was restored by 90%, the corneal defect is not visualized, and there was complete recovery of visual ability (Fig. 5).

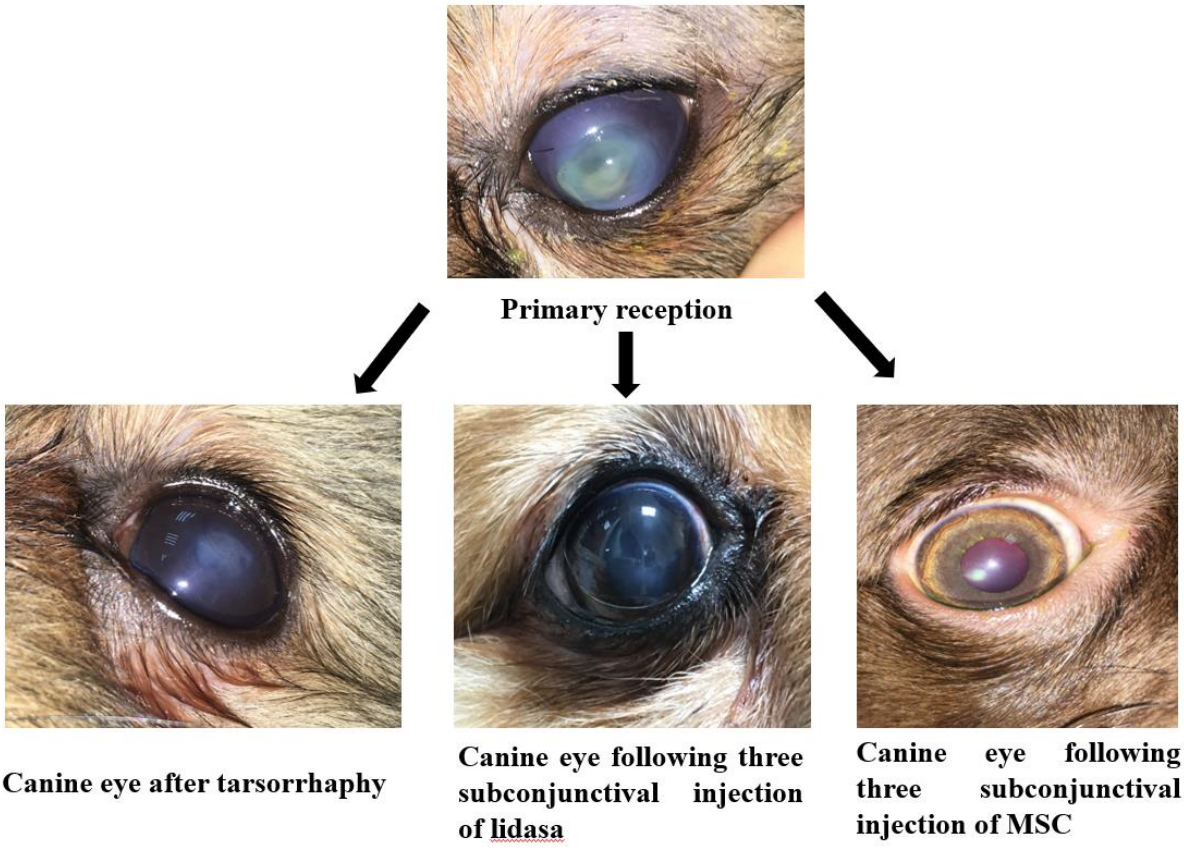

Fig. 5. Results following therapeutic treatment of ulcerative keratitis in dogs using different methods

Discussion

Ulcerative keratitis in dogs is characterized by the expression of different symptoms including: blepharospasm, superficial and deep vascularization of the cornea, hyperemia and chemosis of the conjunctiva, lacrymation, and the main characteristic symptom is damaged integrity of the stratified flat epithelium (Leis & Sandmeyer, 2019). An increase in lacrymation at the acute period of inflammation and a decrease in the amount of lacrymation in the chronic period leads to decreased ocular protective mechanisms (Grahn *et al.*, 2014). All these symptoms were observed into our patients. The results demonstrated the efficiency of introducing MSC as a subconjunctival injection in the subacute stage of ulcerative keratitis in dogs. There are data available indicating that MSCs could be used for the treatment of ulcerative keratitis in dogs with chronic diseases leading to delayed healing times of the ulcers (Choi *et al.*, 2021). This data is topically because according to the literature 75% of dogs with ulcerative keratitis have a concomitant systemic disease (Jones *et al.*, 2022).

The Lan's studies demonstrated, that MSCs was in specifically to the injured cornea and promoted regeneration, highlighting the therapeutic implications of MSC-mediated tissue repair in corneal injury in mouse (Lan *et al.*, 2012). MSCs have shown treatment efficacy for different ocular surface disease types in animals (Zakirova *et al.*, 2019; Zakirova *et al.*, 2022). The mechanisms behind their treatment success remain largely unclear. Several possible mechanisms have been proposed. One of them is synthesis and secretion of a lot of biological activity substances by the MSCs (Park *et al.*, 2018; Lombardi *et al.*, 2019). MSCs have also demonstrated their anti-inflammation and anti-angiogenesis characteristics in corneal chemical burn models. After transplantation MSCs decreased pro-inflammatory cytokines (IL-2, MMP2, IFNγ) and increased anti-inflammatory cytokines such as IL-6, IL-10, TGFβ into the recipient (Zhang *et al.*, 2015).

Another MSCs mechanism during regeneration has been described by several authors. They showed differentiation of MSCs into corneal epithelial cells after being transplanted into injured corneas. Meanwhile after transplantation, the MSCs had no toxic effects on other organs or on resident corneal cells (Sharma *et al.*, 2021). Also Falcao's studies demonstrated that MSCs promoted reduction of inflammation, mobilization of endogenous stem cells and increased concentration of growth factors when treating corneal damages stimulating a rapid recovery, as occurred in a large part of the study animals (Falcão *et al.*, 2019).

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 (Jiang *et al.*, 2010; Yao *et al.*, 2012; Joyce *et al.*, 2012).

Therefore, it is necessary to further investigate and elucidate the mechanisms of action of MSCs on healing in ulcerative keratitis.

Our findings showed that allogeneic adipose-derived MSCs could be applied in veterinary ophthalmology. Subconjunctival injections of adult allogeneic MSC from adipose tissue was clinically safe for use in dogs during the follow-up period. This contributed to the improvement of the clinical manifestations of ulcerative keratitis in dogs, as evidenced by a decrease the intensity and area of the affected areas of the cornea without the use of any combination of drugs. Therefore, MSCs transplantation is an easy and safe alternative method of regeneration in the damaged cornea.

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