Peri-lacrimal gland injection of allogenic adipose-derived mesenchymal stem cells compared to tacrolimus eyedrops in the treatment of canine keratoconjunctivitis sicca

by

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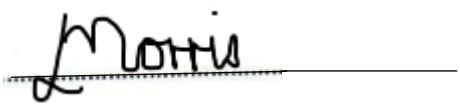
Declaration

I, Laurie Megan Morris, student number 16117574 hereby declare that this dissertation, "Peri-lacrimal gland injection of allogenic adipose-derived mesenchymal stem cells compared to tacrolimus eyedrops in the treatment of canine keratoconjunctivitis sicca", is submitted in partial fulfilment of the requirement for the degree Master of Science (Veterinary Science), in the department of Companion Animal Clinical Studies, Faculty of Veterinary Sciences, University of Pretoria, is my own original work and has not been previously submitted by me for a degree at any other university. All sources that have been cited or quoted within this research paper are both indicated and acknowledged with a comprehensive list of references.



L.M. Morris

Date: 14/02/2024

Dedication

This is dedicated to my late Grandpa Joe. If I become half as much of a success in my field as he was in his, I know I'll have made it.

Acknowledgements

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Abstract

Objective

To describe and compare the efficacy in treatment between a single peri-lacrimal gland injection of adipose-derived mesenchymal stem cells (Stem Cells group) and a twice-daily application of tacrolimus eyedrops (Tacrolimus group) in client-owned dogs suffering from unilateral or bilateral early-stage immune-mediated keratoconjunctivitis sicca (KCS).

Animals Studied

Twenty-two, client-owned dogs (44 eyes), suffering from presumed unilateral or bilateral immune-mediated keratoconjunctivitis sicca (KCS) were enrolled in the study. The immune-mediated aetiology was made by exclusion, with animals suffering from unilateral or bilateral disease with no concurrent systemic disease or neurological deficits. The inclusion criteria were dogs of any age or breed with unilateral or bilateral, immune-mediated KCS (symptoms consistent with KCS and a Schirmer Tear Test type 1 [STT-1] ≤ 15 mm/min; confirmed on examination and previous history); with no co-existing ocular pathologies; and no previous history of treatment, including tear replacements or artificial tears. Exclusion criteria were dogs with bilateral end-stage KCS (advanced corneal pigmentation, fibrosis and/or recurrent corneal ulceration with possible corneal perforation and blindness), as well as the presence of concurrent systemic disease and medications.

Procedures

The dogs were followed for a 2-month period beginning at Day 0, with subsequent follow-ups at Day 30 and Day 60. Dogs were assigned to the various treatment groups based on owner preference. Dogs in the Stem Cells group received a one-time, bilateral, peri-lacrimal gland injection under heavy sedation using a commercially available mesenchymal stem cell solution (5 million viable MSC/mL; 2 mL per vial; VetRenew; South Africa). Dogs in the Tacrolimus group received a twice-daily application of eyedrops using a standard tacrolimus 0.02% eyedrop solution with the same oil carrier, from the same compounding pharmacy and batch. With investigators not being masked to the treatment groups, STT-1 and Tear Break Up Time (TBUT) were recorded, and corneal health subjectively scored using an author-derived simple descriptive scale (0: best; 5: end-stage cornea). Data for each eye was classified as either healthy (STT-1 > 15 mm/min) or having KCS (STT-1 ≤ 15 mm/min), then data within each classification were compared between treatments using a mixed effect model (fixed effect: time, treatment; random effect: dog, eye) using the following interactions: treatment, time, and treatment x time. Significance was interpreted a $P < 0.05$. All data is reported as mean (95% confidence interval of the mean).

Results

Dogs included in the study had a mean (min; max) of 8.7 (1.5 ; 14.0) years, with no difference between treatment groups. A total of 22 dogs began the study, however, 17 dogs (9 in Stem Cells group and 8 in

Tacrolimus group) completed the study. One dog in Stem Cells group and 2 dogs in Tacrolimus group were excluded from the study for not meeting STT-1 inclusion criteria, and 2 dogs in Tacrolimus group were lost at the 60-day follow up.

In KCS eyes (n = 26), STT-1 before treatment Day 0 in were 11 (9, 13) and 11 (8, 14) mm/min for Stem Cells and Tacrolimus, respectively. The STT-1 increased in both treatment groups over time and measured as 18 (16, 21) and 19 (15, 22) mm/min for Stem Cells and Tacrolimus at Day 30, respectively (both $P < 0.001$). The STT-1 stabilised at Day 60 with values of 18 (15, 22) and 19 (15, 23) mm/min for Stem Cells and Tacrolimus, respectively (both $P < 0.001$). The TBUT at Day 0 were 21 (11, 32) and 18 (12, 25) seconds for Stem Cells and Tacrolimus, respectively. The TBUT increased in both treatment groups over time and measured as 23 (16, 30) and 27 (21, 33) seconds at Day 30, and at Day 60 were 36 (27, 44) and 30 (22, 39) seconds for Stem Cells and Tacrolimus, respectively (both $P < 0.001$). Corneal scores improved over time (i.e., healthier corneas) and were significantly different to Day 0 at Day 30 ($P < 0.001$) and Day 60 ($P < 0.001$) for both treatment groups but not different between groups.

Conclusion

A single peri-lacrimal gland injection of adipose-derived mesenchymal stem cells may be an effective treatment in dogs with early-stage immune-mediated KCS and demonstrated similar outcomes as twice-daily application of 0.02% tacrolimus eyedrops over a 60-day period.

List of Abbreviations

KCS	Keratoconjunctivitis Sicca
STT-1	Schirmer Tear Test type 1
TBUT	Tear Break-up Time
IOP	Intraocular Pressure
AD-MSC	Adipose-Derived Mesenchymal Stem Cell
MSC	Mesenchymal Stem Cell
VFAH	Valley Farm Animal Hospital
PTF	Precorneal Tear Film

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Chapter 1 Introduction

As humans have domesticated the canine species over hundreds of years, this has allowed for a more in-depth observation and study of the canine globe. With multiple roles that dogs have in differing spheres, residing as working animals in the field or those merely for companionship, we as humans want whatever is best for them, provided by modern day veterinary medicine.

Over the past three decades, numerous studies have provided evidence that human interactions with companion animals contribute to psychological well-being, recovery from serious conditions, and good health (Walsh, 2009). Overall, human-companion animal interactions increase the amount of neurochemicals in the body associated with bonding and relaxation as well as enhance human immune system function (Charnetski et al., 2004).

Eyesight is a critical component for working dogs, particularly for those that assist their human counterparts with sensory tasks, such as guide dogs and assistance dogs trained to alert and navigate safely (Singletary and Lazarowski, 2021). The canine visual ability is believed to be specialized for motion detection rather than acuity, with increased function in low-light conditions (Alexander and Davidson, 2006; McGreevy et al., 2004).

The goal of medical treatment is to provide animals with optimal vision for their individual circumstances. One disease that challenges this goal is keratoconjunctivitis sicca (KCS). Undoubtedly, the chronicity of each case must be considered, as patients with advanced epithelial keratinization and scarring will have diminished visual function. However, due to the importance of these animals in society and their owners' dedication to their well-being, this treatment is a viable option.

Major advancements in veterinary ophthalmology have given many patients a new lease on life, alleviating the ailments that afflict them. Whether the treatment be surgical or medical, the outcome for our eleven-thousand-year-old domesticated pets has demonstrated to be astounding. Tacrolimus, an immunosuppressive drug first discovered in 1984 (Wallemacq and Reding, 1993), is an effective drug in the treatment of keratoconjunctivitis sicca (KCS) today, while cyclosporine remains the mainstay treatment in most countries. However, newer remedies might prove equally beneficial.

Chapter 2 Literature Review

2.1 The Canine Corneal Structure

The outer surface of the cornea is convex and smooth, with the moisture emanating from the precorneal tear film (PTF), with the inner surface being concave and concurrently forming the anterior border of the anterior chamber of the eye (Spreull, 1966). The normal, healthy cornea is avascular, receiving nourishment from the limbal capillaries, precorneal tear film (PTF), and aqueous humor (Spreull, 1966). The majority of the corneal oxygen requirements are supplied by the epithelium which takes it from the surrounding air, as opposed to when the eyelids are closed, the oxygen requirements are derived from the capillaries of the palpebral conjunctiva (Spreull, 1966).

The cornea is comprised of 4 layers in the dog, with a thickness of 562 ± 6.2 µm (Gilger et al., 1991). The outermost epithelial layer of cells along with its basement membrane, the stroma, Descemet's membrane, and the innermost endothelial layer of cells. It forms a unique translucent boundary between the outer environment and the intraocular tissues. Loss of this corneal translucency runs parallel with corneal diseases (Gelatt, 2002).

The epithelial layer has a rapid repair rate when injured (Spreull, 1966). The stromal layer forms the majority of the cornea (Spreull, 1966). Following an injury, a long-lasting corneal opacity often develops due to the formation of a fibrous scar (Spreull, 1966). The Descemet's membrane is an acellular, well-formed, homogenous elastic structure that is recognizable from the stroma (Spreull, 1966). Finally, the endothelium consists of a single layer of flattened cells interconnected by extensive interdigitations (Spreull, 1966). When injury ensues, the endothelial cells migrate into and within the stromal layer whereby they essentially assist in rebuilding the Descemet's membrane (Spreull, 1966).

2.2 The Precorneal Tear Film

The PTF constitutes a trilaminar fluid consisting of 3 components and measuring approximately 7-10 µm which make contact with both the cornea and conjunctiva (Ribeiro et al., 2008). The PTF is responsible for the nutrition and nourishment of the cornea, assists in leukocyte distribution, lubrication of the eyelids and ocular surface as well as cleaning of the latter (Ribeiro et al., 2008). The meibomian glands (Figure 2.1) and nictitating lacrimal glands (Figure 2.2) and conjunctival goblet cells produce the lipids, aqueous and mucin layers of the PTF, respectively (Best et al., 2014). Each layer of the PTF provides a crucial role in corneal health and functioning (Table 2.1). Under natural circumstances, tear production declines with age in dogs (Hartley et al., 2006). The lacrimal gland location lies over the superotemporal part of the globe, specifically in the dog, beneath the orbital ligament and supraorbital process of the frontal bone and is also related to the medial surface of the zygomatic bone (Cooper, 2012).

Table 2.1 The various functions of the different layers of the precorneal tear film (Best et al., 2014).

Type	Function
Aqueous	○ Removes bacteria and waste material ○ Provides surface lubrication, smooths the surface for optical clarity and corneal nutrition ○ Non-specific antimicrobial substances: lysozyme, lactoferrin, α-lysine, and complement ○ Specific antimicrobial substances secretory immunoglobulins A, G and M. ○ Toll-like receptors are expressed by the corneal and conjunctival epithelial cells which play a role in the defence against many types of microbial infections
Lipid	○ Provides surface tension to prevent tear film overflow ○ Limits evaporation ○ Binds the tear film to the cornea
Mucin	○ Enhances the spread of the tear film ○ Makes the corneal surface which is normally hydrophobic more hydrophilic to permit spreading

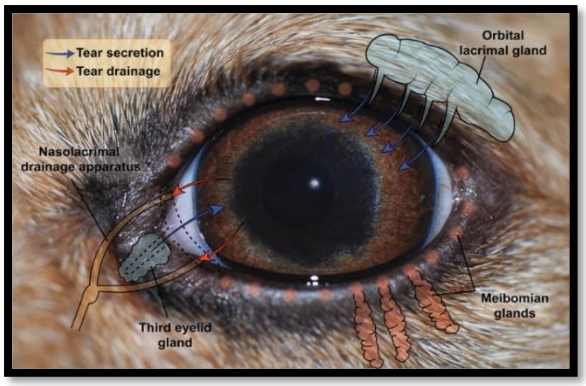


Figure 2.1 Depiction of canine meibomian glands (Sebbag, 2020) (from John Wiley and Sons)

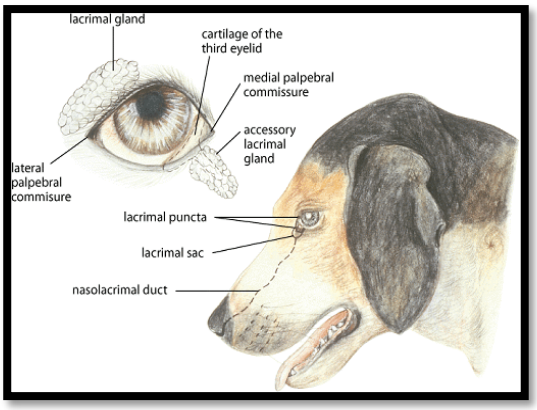


Figure 2.2 Depiction of canine orbital and nictitating lacrimal glands (from MSD Veterinary Manual, https://www.msdvetmanual.com/eye-diseases-and-disorders/ophthalmology/nasolacrimal-and-lacrimal-apparatus)

The outermost lipid layer forms a thin oil cover of approximately 0.05-0.1 µm in thickness (Ribeiro et al., 2008). The intermediate aqueous layer makes up the largest proportion of the tear film, measuring at a thickness of 7.0 µm (Ribeiro et al., 2008). The deepest and innermost mucin layer is approximately 1.0 µm thick, and is produced by goblet cells of the conjunctival fornix (Ribeiro et al., 2008). However, more recent studies have shown that the distinction between the layers is not so clear-cut and not so rigid in correctness due to the strict affiliation among the proteic substances that make up the three layers (Gipson et al., 1992). The glycocalyx is extracellular, secreted by superficial corneal epithelial cells and is bound to the apical microvilli, as well as in an intimate affiliation with the mucous layer (Bock and Hanak, 1971; Latkovic and Nilsson, 1979; Mishima, 1965; Nichols et al., 1983). With respect to excretion of the tear film, it encompasses a drainage system starting in the lacrimal puncta, the lacrimal canaliculi; next the lacrimal sac and lastly, the nasolacrimal duct (Grahn and Storey, 2004). Tears, as a whole, contain growth factors, namely nerve growth factor, lysozymes, immunoglobulins along with other components that provide antibacterial action (Dodi et al., 2009; Hartley et al., 2006; Ofri et al., 2009).

2.3 Control of Lacrimal Gland Secretion

Secretion from the lacrimal gland is under both hormonal and neural control (Williams, 2008). The neural control has both sympathetic and parasympathetic innervation with a reflex arc identified from sensory nerves found in the cornea (Williams, 2008). These, in turn, activate both efferent sympathetic and parasympathetic nerves which originate in the parasympathetic motor nucleus of the facial nerve but travel together with the trigeminal nerve to the lacrimal gland (Williams, 2008) resulting in its subsequent functioning. The neurotransmitters acetylcholine, which is part of the parasympathetic nervous system; and noradrenaline, which is part of the sympathetic nervous system, are the most

influential stimuli with respect to electrolyte and water secretion in tears (Lamberts, 1994). Hormonal functioning is controlled by means of the hypothalamopituitary-gonadal axis which has been found to have a large influence and effect on tear secretion (Williams, 2008). These hormones, namely, α-melanocytic-stimulating hormone, prolactin, oestrogens, adrenocorticotrophic hormone, progestogens and androgens, all have an effect on lacrimal gland functioning (Lamberts, 1994), with androgens, in particular, being the most potent stimulators of tear production (Williams, 2008). The main consequences of lacrimal gland inflammation include loss of acinar epithelial cells, inadequate tear production and secretion, an increased production of proinflammatory cytokines along with the presence of immune cell infiltrates (Zoukhri, 2010). Animal models have also shown that proinflammatory cytokines that are produced inhibit the release of neurotransmitters which results in insufficient lacrimal gland secretion (Zoukhri, 2010).

2.4 Overview of KCS

Keratoconjunctivitis sicca, more generally known as dry eye, is an inflammatory condition of the ocular surface. It is a frequently occurring condition, with a current prevalence of between 4% and 20% in the dog (Barabino and Dana, 2004), that is important to diagnose and treat, as in severe cases, it can be painful and ultimately lead to blindness or loss of the eye in untreated cases (Cooper, 2012). Dry eye syndrome (DES) can be divided into 2 types. The first being quantitative tear film deficiency, also known as KCS in which the aqueous portion of tear production is deficient whereby clinical signs are caused by a pathological decrease in, specifically, the aqueous layer of the tear film (Williams, 2008). In the second type of DES, or qualitative tear film deficiency, tear evaporation is the main reason for the tear deficiency (Williams, 2008). This type of DES occurs when there is chronic inflammation of the eyelids or the conjunctiva, decreased goblets cells or an abnormal mucous layer. It is often seen in brachycephalic breeds in which lagophthalmos, the failure of complete eyelid closure, results in tear film instability, or when Meibomian gland dysfunction results in lower lipid secretion and therefore increased tear loss through evaporation (Williams, 2008). The former type of KCS is the leading cause of chronic and recurrent canine conjunctivitis, affects approximately 1.5% of dogs and is often confused for bacterial conjunctivitis (Kaswan and Salisbury, 1990). However, facial paralysis resulting in cessation of eyelid motor function, trigeminal nerve palsy resulting in a lack of sensation to the cornea as well as third eyelid and eyelid disorders will all also cause an inadequate spread of the tear film (Cooper, 2012).

2.4.1 Aetiologies and Predispositions of KCS

There are many proposed aetiologies of KCS in dogs, such as:

1) Infectious as caused by leishmania or canine distemper virus (Berdoulay et al., 2005; De Almeida et al., 2009; Ciaramella et al., 1997; Naranjo et al., 2005).
2) Neurogenic which can present as an acute and severe dry eye with ipsilateral dry nose (xeromycteria) (Matheis et al., 2012) (Galley et al., 2022).

3) Congenital which has a genetic component known as congenital alacrima seen in the Cavalier King Charles Spaniel, Bedlington Terrier, English Cocker Spaniel, and Yorkshire Terrier (Herrera et al., 2007; Westermeyer et al., 2009; Sanchez et al., 2007).
4) In the Cavalier King Charles Spaniel, KCS can also be associated with ichthyosiform dermatosis (dry eye curly coat syndrome) (Barnett, 2006; Hartley et al., 2012b; Hartley et al., 2012a).
5) Drug induced KCS may be permanent or temporary according to the toxic effects on the gland (Dodi, 2015a). Temporary KCS can be caused by sedatives, atropine or due to local or systemic anaesthetic administration (Soontornvipart et al., 2003), with a general return to normal conditions being achieved if the use of the agent is interrupted (Dodi, 2015a). Permanent drug induced KCS can be seen with the use of sulphasalazine (Barnett and Joseph, 1987).
6) Radiation therapy may cause damage to the lacrimal glands but this is a less common cause of KCS (Maggs et al., 2017).
7) Metabolic conditions such as hypothyroidism and diabetes mellitus (Miller and Panciera, 1994; Cullen et al., 2005) may also induce KCS.
8) Iatrogenic causes, due to the surgical removal of the third eyelid gland, in patients suffering from 'cherry eye' (Saito et al., 2001) may result in iatrogenic KCS. The reason for this being that the third eyelid gland is responsible for 30% of tear production, while the main orbital lacrimal gland is responsible for producing the rest (Helper, 1996; Gelatt et al., 1975). In many instances, quantitative KCS is temporary but leads to long term qualitative KCS with an unstable PTF (Saito et al., 2001).
9) Lastly, an immune-mediated cause is the most prevalent form in dogs (Dodi, 2015b). It is a diagnosis in dogs that is considered relatively common; however, paradoxically often overlooked (Best et al., 2014).

The causes of KCS are so many that oftentimes the exact etiology remains undetermined (Gao et al., 1998).

The following factors tend to be used to aide in a diagnosis KCS when a dog presents with ocular pathology:

1) There are certain predisposed breeds such as: Pugs, Samoyeds, American Cocker Spaniels, English Bulldogs, Pekingese, Miniature Schnauzers, Bloodhounds, Boston Terriers, English Cocker Spaniels, Shih Tzus, Cavalier King Charles Spaniels, Lhasa Apsos, West Highland White Terriers, English Springer Spaniels and Yorkshire Terries (Cooper, 2012; Dodi, 2015a).

2) Being female is a predisposing factor due to prolactin inhibiting tear production. Barnett (1988) reported that the greater number of cases of dogs suffering from KCS occurred in females rather than males, and notably in the West Highland White Terrier.
3) Dogs with certain endocrinopathies such as diabetes mellitus, hyperadrenocorticism and hypothyroidism have been shown to have reduced tear production.

2.4.2. Clinical Signs of KCS

The inflammatory changes in the KCS condition leads to a significant reduction in tear production with the following propositions as to how it occurs:

1) The lymphocyte-associated cytotoxicity of the lacrimal tissue is the central cause of the pathologic effects on lacrimation
2) Apoptosis of glandular epithelial cells is crucial in tear hyposecretion
3) Cytokine release from inflammatory cells alters tear production
4) Inflammatory cells and their associated cytokines influence neurotransmitter function in the lacrimal gland thereby inhibiting the neurologic stimulation of tear secretion (Williams, 2008).

Reduction in overall precorneal tear film production results in the following sequelae:

1) Secondary ocular infections
2) Malnutrition and dehydration of the conjunctival and corneal epithelium
3) Chronic inflammation of the ocular surface as a result of increased surface friction (Best et al., 2014).

Dogs with KCS often present with congestion of the conjunctiva, ocular discomfort and corneal opacities which may eventually lead to blindness.

The following are classic and distinguishing features of KCS:

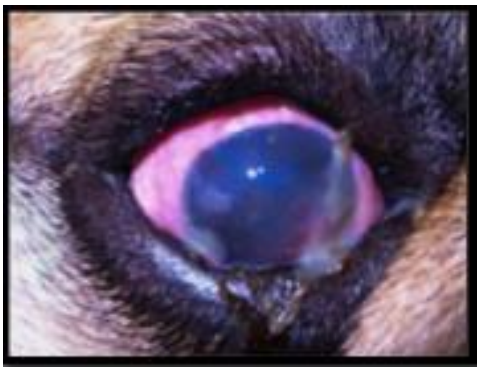
1) Conjunctivitis, which is the consistent component of KCS (Kaswan, 1994). Conjunctivitis is not typically noted to be painful, but rather more irritative with dogs responding to this by rubbing their eyes against objects and their paws (Kaswan, 1994)
2) Blepharospasm due to ocular pain which is seen initially but will decrease over time due to a degradation in nerve endings (Cooper, 2012). Discomfort is a highly variable feature in dogs suffering from KCS, with the apparent discomfort being contrary to apparent clinical signs (Kaswan, 1994)
3) A dull overall appearance to the cornea (Cooper, 2012)
4) Mucoid to mucopurulent ocular discharge (Dodi, 2015b)
5) Hyperaemia of the surrounding conjunctiva (Dodi, 2015b)
6) Secondary bacterial infections due to decreased ocular defence mechanisms (Dodi, 2015b).

7) Signs of keratitis with variable degrees of corneal fibrosis, melanosis, oedema and neovascularization

Advanced stages of the condition include corneal pigmentation, ulceration, neovascularization and epithelial hyperplasia (Dodi, 2015b). Blood vessels and inflammatory cells infiltrate into the anterior corneal stroma, with the presence of these factors resulting in potential vision loss for the affected patient (Best et al., 2014). Blindness can become a complaint in patients suffering from KCS due to the corneal scarring and advanced pigmentation resulting in vision loss (Kaswan, 1994), after several years of active disease and in extremely advanced cases. Keratitis tends to occur in most cases, however, severe cases of KCS result in severe keratitis whereby the corneal hypertrophy can become so extreme so as to result in lagophthalmos which further compounds the effects of tear deficiency (Kaswan, 1994). Corneal ulceration becomes apparent as a result of desiccation occurring most commonly in the region of the central cornea, with melting ulcers occurring occasionally (Kaswan, 1994).

Owing to the beginning stages of KCS appearing as a conjunctivitis, it is often misdiagnosed as a plain bacterial infection and is treated with antibiotics (Dodi, 2015b). With only temporary improvements, the dry state of the affected eyes show limited improvements in condition due to the mere use of the antibiotic and or corticosteroid eyedrops (Dodi, 2015b). However, once the therapy has come to an end, the condition will return to its original state, or sometimes, be worse than it was at the beginning (Dodi, 2015b). Refer to Table 2.2 for staging and progression of clinical signs of canine KCS.

Table 2.2 Depiction of the stages in clinical signs due to chronicity of keratoconjunctivitis sicca in the canine eye (Dodi 2015a)

Stage	Clinical Signs	Image
Initial	• Blepharospasm • Mucopurulent ocular discharge • Conjunctival hyperaemia	
Intermediate	• Corneal opacification • Intense mucoid ocular discharge • Corneal vascularisation	
Final	• Pigmentation • Fibrosis • Recurrent corneal ulceration with possible corneal perforation and blindness. However, corneal ulceration can occur at any stage of KCS	

2.4.3 Diagnostics of KCS

Owing to easily identifiable symptoms and diagnosis by non-invasive procedures, corneal diseases are commonly diagnosed.

The basis of KCS diagnostics require classic and distinguishing clinical sign and an indication in reduction of the aqueous portion of tear production, measured using the Schirmer Tear Test (STT) (Lewin, 2014). The STT was initially experimented by Otto Schirmer, a German ophthalmologist, around one century ago (Schirmer, 1903). There are 2 types of STT's that can be performed in clinical practice, however, in our study, only STT-1 was performed. STT-1 measures both basal and reflex tear production, with no topical anaesthetic being applied preceding the start of the test. STT-2 measures only basal tear production, with topical anaesthetic being applied to the cornea preceding application of the test strip (Lewin, 2014). Table 2.4 summarises a general overview of the categories of KCS based on STT-1 values.

Table 2.3 Depiction of the categories of KCS based on Schirmer Tear Test-1 values (Haeussler Jr, 2019)

Degree of severity	Measurement (mm/minute)
Normal	>15
Subclinical	11-14
Mild	6-10
Severe	5 or less

According to Herrera (2005), the KCS condition begins before the STT values become abnormal; while the qualitative changes in the tears can be attributed to the first inflammatory changes in the lacrimal glands albeit with normal or slightly decreased STT-1 values (Herrera, 2005).

Owing to corneal ulceration being a frequent co-morbidity in dogs with KCS, it is vital that both eyes be tested for concurrent corneal ulceration, using fluorescein stain. Furthermore, a Tear Break Up Time (TBUT) is assessed using fluorescein stain, with normal values ranging between 15 and 25 seconds compared to values of 10 seconds or less being indicative of tear film instability (Lewin, 2014). TBUT is becoming a more common test to assess the combination of increased evaporation and reduced tear production in ocular surface tear film deficiency (Saito and Kotani, 2001). Lissamine green is complementary with BUT, (Smith et al., 2020), however, they look at different aspects of the ocular surface homeostasis.

The immune-mediated form of KCS is primarily accepted as the most prevalent of all possible forms (Gelatt, 2013; Roszkowska et al., 2021). The condition is often bilateral (Kaswan et al., 1985) but early cases may present unilaterally, with lymphocytic infiltration, which was noted using histopathology in this instance, is the characteristic inflammation, and the resultant damage to the lacrimal gland diminishes tear production and consequently, the aqueous tear film (Kaswan et al., 1984; Izci et al., 2015). Subsequently, the ocular surface becomes desiccated and inflamed and the vascularisation that develops eventually scars the cornea, resulting in vision loss for the dog (Gilger et al., 2013; Sanchez et al., 2007).

2.5 Treatments for KCS

Medical therapy aims to eradicate the cause of the condition when possible, control and prevent secondary bacterial infections, to stimulate tear production, reactivate the tear film, as well as to reduce inflammation (Dodi, 2015a). The current treatment protocols involved in the remedy of KCS are in the form of eyedrops and they fluctuate in their safety and efficacy, are challenging to administer and the treatment tends to be lifelong (Worster et al., 2000; Lemp, 2008). They may also be ineffective in some patients (Williams, 2008; Zhou and Wei, 2014; Barachetti et al., 2015).

With respect to topical eyedrop therapy, lacrimomimetics are substances containing moisturising substances with the aim of replacing one or more components of the tear film that are known to be deficient (Ribeiro et al., 2008). These are used as adjuvant topical treatment therapy to lacrimostimulants and are usually used as palliative treatment to relieve the dry eye condition (Ribeiro et al., 2008). Lacrimostimulants are drugs that have the ability to stimulate tear production and include immunosuppressive/anti-inflammatory agents (Ribeiro et al., 2008). In a study conducted by Barabino et al., (2020), a consensus was proposed for a new classification of eyedrops that are used to improve ocular surface epithelia and tear film (Barabino et al., 2020). The new proposed terms are “multiple-action tear substitutes”, “wetting agents” or “ocular surface modulators”, and this was done in order to characterise eyedrops use to enhance the ocular surface epithelia and tear film, along with the new definition of Dry Eye Disease, which observes the loss of ocular homeostasis (Barabino et al., 2020). According to Dodi (2015a), stimulation of natural tear production seemingly results in the highest resolution of clinical signs as well as the prevention of vision loss in affected patients.

Current therapies for the immune-mediated form of KCS include immune-suppressive therapies (Sanchez et al., 2007; Su and Yang, 2020), such as cyclosporine A (CsA) (Colligris et al., 2014; Radziejewski and Balicki, 2016), tacrolimus (Berdoulay et al., 2005; Nell et al., 2005), or pimecrolimus (Nell et al., 2005). The application of short-term corticosteroids is a possibility in improving a patient’s clinical signs, but should be used with discretion due to the potential occurrence of corneal ulceration (Dodi, 2015b). In the same instance, immunosuppressive topical medication may result in irritation of the eyes (Hermida-Prieto et al., 2021).

In addition to the lacrimostimulants and lacrimomimetics used, topical steroids can be used, provided no ulceration is present, which lessen the overall amount of vascularisation, potential pigmentation and hyperaemia (Cooper, 2012). Topical antibiotics can be used to treat secondary bacterial infections if mucopurulent discharge is present (Cooper, 2012). Mucolytics, such as the use of acetylcysteine, can occasionally be useful in the beginning phases of disease progression to remove excessive amounts of mucin (Cooper, 2012).

Tacrolimus is a macrolide calcineurin inhibitor immunosuppressant drug isolated from *Streptomyces tsucrenaeseis* (Ribeiro et al., 2008). Calcineurin is a protein phosphatase that is required for T-lymphocyte activation (McShane et al., 2016; Wallemacq et al., 2009). Tacrolimus binds to a specific immunophilin, FK506, with its mechanism of action being similar to that of cyclosporin (Moore, 2004). It has been the standard treatment in patients suffering from KCS for many years owing to the majority of them suffering from an immune-mediated component. According to Kalt (2017), its usage today has surpassed that of topical cyclosporine. However, it does depend greatly on where one practices within the world. Although tacrolimus and cyclosporine have a similar mechanisms of action, tacrolimus is a more potent immunosuppressant, with a 10- to 100- fold greater *in vitro* immunosuppressive activity in comparison to cyclosporine (Armstrong and Oellerich, 2001). Along with its greater potency, therapeutic whole blood trough concentrations for tacrolimus are about 20-fold lower than the comparable concentrations for cyclosporine (Armstrong and Oellerich, 2001). Additionally, the range between subtherapeutic and toxic concentrations of cyclosporine is narrow (Armstrong and Oellerich, 2001). However, systemic tacrolimus administration can result in more severe side effects than cyclosporin, such as nephrotoxicity (Moore, 2004).

Tacrolimus, at a concentration of 0.02%, is a topical eyedrop formulation, applied twice-daily and is often indicated as lifelong treatment. In the author's experience, some dogs seem to react to the application of the eyedrops and we speculate that it is because of a stinging or burning sensation. With chronic and consistent use, it helps to reduce the affected cornea of blood vessels and pigmentation as well as stimulating tear production. However, due to chronic use, monthly costs can build up and can become quite cost prohibitive. In South Africa, two types of tacrolimus formulations are available for use. An oil-based eyedrop and an aqueous-based eyedrop. In a study conducted by (Zulim et al., 2018), diluting tacrolimus eyedrops in olive oil or linseed oil were effective in the treatment of KCS with linseed oil being potentially considered as an alternative diluent for tacrolimus eyedrops. The oil-based drop costs around R500 and the aqueous-based drop costs around R470 with each bottle lasting approximately 1 month. While being an efficient form of treatment for many years, decreased owner compliance, mainly due to poor patient compliance can be the main concern with treatment and thus improvements in condition may not be favourable.

Kaswan (1994) reported that the topical application of CsA, as a treatment of KCS, leads to a gradual recovery in lacrimation in 80% of affected dogs. In that study, it also noted that with longer term application, conjunctivitis and keratitis is reduced in patients and this is largely owed to CsA's anti-inflammatory activity (Kaswan, 1994). Overall, CsA stops the inflammatory process thereby diminishing corneal and conjunctival lesions, recovers eye lubrication and halts lacrimal gland aggression (Dodi, 2015b). Cyclosporine, however, has less efficacy when used in advanced cases of canine KCS and can also cause hypersensitivity to the eyes (Wei et al., 2022).

Cyclosporine's use however, has been surpassed by that of tacrolimus. As is the concern with tacrolimus, owner responsibility is key due to daily application and owner compliance becomes a concern. In addition to this, the ongoing expense of treatment become limiting factors. In another study conducted by Barachetti et al., (2015), solid, silicone matrix episcleral implants that were designed to release cyclosporine at therapeutic concentrations to the ocular surface were used for an extended period of time in dogs. For those animals that were implant recipients, at a 6-month follow-up, STT-1 scores of more than 10 mm/min were maintained and the topical cyclosporine treatment could be discontinued which proved these implants to be beneficial. This mode of KCS treatment may prove favourable in those less compliant owners due to the implant lasting approximately 6-10 months but would require replacement.

In addition, a downside to chronic topical immunosuppressive therapy for KCS creates the opportunity for potential complications to develop such as corneal squamous cell carcinoma as well as conjunctival and corneal infections (Swinger et al., 2009; Dreyfus et al., 2011).

Another form of treatment that are not focused on daily eyedrops is stem cell therapy. Mesenchymal stem cells (MSC) are drawn to injured tissue while too provide anti-inflammatory, trophic, immunomodulatory as well as regenerative effects (Caimi et al., 2010; Tolar et al., 2010). MSC are multipotent cells with the capacity to differentiate into diverse cell lineages along with secreting different bioactive molecules with immunomodulatory, trophic and paracrine functions (Murphy et al., 2013; Kapur and Katz, 2013; Carrade and Borjesson, 2013). Adipose-derived MSC (AD-MSC) are perceived as the most promising stem cell type with regards to stem cell therapy owing to the simple procedures required for their harvesting, isolation and high cell yield during culturing (Voga et al., 2020). Adipose tissue is considered a plentiful source for acquiring MSC with a large proliferation capacity along with them being conveniently accessible (Villatoro et al., 2015). In a study noting that canine AD-MSC can be collected from a small amount of adipose tissue (Neupane et al., 2008), a separate study by Wei et al., (2022) proved this by yielding around 3x10^7 cells in primary culture from 5 mL of adipose tissue. Minimally invasive techniques are required in comparison to the extraction of other stem cell lines, such as bone marrow-derived MSC (Konno et al., 2013), with both local and systemic administration in the treatment of various disorders being feasible (Wood et al., 2012). In dogs,

the collection of adipose tissue can be done during routine surgical procedures, through biopsies, or via adapted liposuction procedures (Vieira et al., 2010). Unlike MSC in humans, there are no standardised and consensus-derived characterisation criteria that are available for MSC from general animal origin (Jiang et al., 2002; Dominici et al., 2006; De Schauwer et al., 2011). However, MSC must stick to plastic, look like MSC under a microscope, and be tested for surface markers such as CD90, CD34 and not express CD45 and MCH II to be accepted as a pure MSC culture.

In a study conducted by Guercio et al., (2013), it was found that stem cell donor age along with the anatomical origin of the adipose tissue have an effect only on the proliferative scope of the MSC (Guercio et al., 2013). Additionally, cells show a progressive decrease in their stemness characteristics with increased numbers of passages, while the finest qualitative and quantitative characteristics are captured at initial passages (Guercio et al., 2013).

It has been proven that MSC show expansive growth *in vitro* from small amounts of collected bone aspirates or from adipose tissue and, if placed into various growth media, have the ability to differentiate into bone, cartilage, fat, myoblasts, as well as potentially neurons and astrocytes (Giordano et al., 2007). MSC, *in vitro*, have the capacity to differentiate into diverse cell lineages as well as secrete different bioactive molecules (Murphy et al., 2013; Kapur and Katz, 2013; Carrade and Borjesson, 2013). However, during *in vivo* treatment, injecting MSC at the site of treatment allows these cells to differentiate according to the locally secreted growth media. In a study conducted by Wood et al., (2012) which investigated an *in vivo* imaging and migration study of MSC, it demonstrated that when MSC's are injected periocularly and intra-articularly, the diminution of the detected signal at the site of injection lessens, proving the migration of cells away from the local site of delivery. Additionally, with MSC from multiple sources showing migration after injection in multiple animal models including both human and dog patients (Barbash et al., 2003; Liechty et al., 2000; Schäffler and Büchler, 2007). Furthermore, *in vivo* results prove that MSC can be administered numerous times without evoking a cellular immune response (Carrade et al., 2011; Wood et al., 2012).

Currently, AD-MSC are being examined for their use as treatment for a variety of disorders including spinal cord injury, immune disorders, diabetes mellitus and skin regeneration (Wood et al., 2012; Djouad et al., 2009; Zuk, 2010; Ben-Ami et al., 2011). They too possess immunomodulatory properties, by having the ability to modulate both adaptive as well as innate immunity (Ben-Ami et al., 2011). While not completely understood, MSC alter B-cell function, inhibit T-cell proliferation (Ben-Ami et al., 2011; Corcione et al., 2006), inhibit dendritic cell maturation and differentiation and also downregulate MHC II (Ben-Ami et al., 2011).

Autologous MSC therapy, whereby the stem cells are derived from and treated within the same patient, in dogs suffering from ocular pathology has been reported on. Limbal stem cells used in the eye had a

vital role in maintaining and sustaining the integrity of the surface of the cornea; whether it be in a healthy condition or after injury, through corneal repair and renewal (Ana Kovsca Janjatovic, 2015).

However, allogeneic MSC, that are derived from the same species animal but are used for treatment in an unrelated patient of the same species, allow for a fast therapy initiation without the need to harvest MSC from each patient (Hermida-Prieto et al., 2021). The use of allogeneic MSC therapy is a possibility owing to their low immunogenicity (Corcione et al., 2006; Tyndall and Uccelli, 2009; Aggarwal and Pittenger, 2005). As previously mentioned, MHC II is downregulated, which is the cause of the low immunogenicity (Gupta et al., 2008; Smith, 2007; de Bakker et al., 2013). Owing to their low immunogenicity but also along with their immunoregulatory potential, the allogeneic use and potential of these stem cells is an encouraging new treatment for autoimmune diseases that are considered severe or refractory to conventional therapies (Murphy et al., 2013; Leto Barone et al., 2013). This low immunogenicity allows for a rapid initiation of therapy without requiring to screen the most suitable donors, examining *in vitro* the MSC profile and immunosuppressive features, preventing the transmission of infectious and contagious diseases and lastly, not requiring to harvest the MSC from each patient needing treatment (Carrade et al., 2011; Wood et al., 2012). Additionally, in a study whereby MSC were used topically in healthy laboratory beagles, subsequent examinations exhibited that repeated peri-ocular transplantations of specifically allogeneic MSC were safe for use (Bittencourt et al., 2016; Park et al., 2013; Wood et al., 2012).

Canine AD-MSC have been used to treat dogs that are affected by bilateral KCS by local implantation into the gland of the third eyelids and lacrimal glands with subsequent positive outcomes (Villatoro et al., 2015; Bittencourt et al., 2016). It has been suggested that canine AD-MSC operate by means of an anti-inflammatory effect, by released anti-inflammatory cytokines and immunomodulatory factors such as TGF-β (Kang et al., 2008; Abughanam et al., 2019; Stevenson et al., 2012). Villatoro et al. (2015) suggested that this effect breaks the cycle in production of proinflammatory cytokines and lacrimal lymphocyte proliferation, while concurrently reactivating tear production to commence the repair of the damaged ocular surface. The potential mechanism of action of AD-MSC in the treatment of KCS is centred around their anti-inflammatory and immunomodulatory scope, stimulated by proinflammatory cytokines such as IL-6, TNF and INF in the process (Stevenson et al., 2012; Massingale et al., 2009), while concurrently through the secretion of immunomodulatory factors such as IDO, HGF, PGE2 and TGF-β (Kang et al., 2008). This effect halts the production of lacrimal lymphocyte proliferation and proinflammatory cytokines, while concurrently reactivating tear production to start ocular surface healing (Villatoro et al., 2015).

In a study conducted by Bittencourt et al., (2016), it was shown that with a singular dose of a low number of MSC (1 million MSC suspended in 0.5 mL of 0.9% NaCl), KCS can be treated in canines. The suspension was partially injected (0.3 mL) into the region of the dorsal lacrimal gland with the

remaining 0.2 mL being injected into the lacrimal gland of the third eyelid (Bittencourt et al., 2016). In comparison to the use of immunosuppressive agents, such as tacrolimus and cyclosporine in the treatment of KCS, MSC injection and transplantation is effective over a longer period of time, 12 months in the case of this study, with a singular treatment administration along with no provision of daily drug administration to the patient (Bittencourt et al., 2016). The STT-1 was the only outcome measured in that study, whereas we also assessed tear quality with TBUT. All eyes within the study before MSC transplantation presented at values lower than 15mm/min. They were divided into a mildly to moderately affected group and a severely affected group. It was found that statistically significant increases in STT-1 values were observed in both groups after MSC transplantation after 28 days (Bittencourt et al., 2016).

In another study conducted by Villatoro et al., (2015), the results of using AD-MSC proved promising when treating animals with severe KCS refractory to conventional eyedrop treatments where no other feasible treatment was available for their ocular pathology. In the same study, none of the animals showed signs of systemic or local complications (Villatoro et al., 2015). With regards to aqueous tear production, STT-1 values increased significantly from baseline at the 3rd month and continued to rise at both the 6- and 9-month evaluations. The last 2 values were significantly different from the baseline. It was found that in spite of this rise between the 3rd and 9th month, there were no statistical differences among these samples (Villatoro et al., 2015). In the study conducted by Hermida-Prieto et al., (2021), all patients within the study suffered from spontaneous, mild/moderate/severe or early/subclinical KCS with STT-1 values ≤14mm/min and found it to be an especially effective therapy in dogs with initial STT-1's between 11-14mm/min (Hermida-Prieto et al., 2021).

2.6 Conclusion of the Literature Review

Allogeneic MSC treatment may prove beneficial for those less compliant owners as daily treatment is not a requirement. A few limiting factors, however, are that MSC local implantation is required to be performed by specifically trained staff, which unfortunately limits their potential use in many veterinary clinics. Additionally, it has been proposed that MSC should ideally be applied in the early stages of KCS, when the disease has not yet had much chance to advance and can still be reversed (Hermida-Prieto et al., 2021). Overall, MSC implantation to treat KCS has not been compared in the same study to the routine approach of using tacrolimus and this warrants investigation.

The use of MSC therapy in the treatment of various other canine conditions, such as osteoarthritis and acute liver failure has proven to show great benefits and success. Allogeneic MSC proved to be an acceptable substitute of therapeutic modality when treating canine osteoarthritis (Shah et al., 2018). While the current treatment methods of KCS prove to still have a successful outcome, MSC therapy may further enhance a beneficial and positive outcome while concurrently causing no harm.

2.7 Research Question, Aim and Objective, and Hypothesis

2.7.1. Research Question

Is a one-time peri-lacrimal gland injection of AD-MSC an effective treatment of KCS in dogs? And how does AD-MSC compare to tacrolimus 0.02% eyedrop therapy in dogs with KCS?

2.7.2 Aim and Objectives

The aim is to classify and compare the treatment of both tacrolimus and AD-MSC in dogs with KCS. The objective is scoring corneal health for resolution of associated clinical signs and to measure STT-1 and TBUT before and after treatment to classify and compare treatments.

2.7.3 Hypothesis

H0 The use of adipose-derived mesenchymal stem cells treatment is no different in resolving clinical signs and normalising tear production and tear quality in KCS dogs compared to the use of tacrolimus eyedrops

H1 The use of adipose-derived mesenchymal stem cells treatment is different in resolving clinical signs and the normalising tear production and tear quality in KCS dogs compared to the use of tacrolimus eyedrops

Chapter 3 Materials and Methods

3.1 Experimental Animals, Ethics and Veterinary Care of Client-Owned Animals

The dogs used in the investigation were those that had to undergo treatment, regardless of the study. Both research and animal ethics approval (REC058-22) was granted for the following trial by the Research and Animal Ethics Committees of the Faculty of Veterinary Science, University of Pretoria. Good practices of medicine, general animal husbandry as well as all trial procedures were strictly adhered to in accordance with the outlined research protocol. The trial was conducted at Valley Farm Animal Hospital (VFAH) and VetVision Animal Eye Clinic, the latter where cases were recruited for the trial. All dogs were treated within the normal standard of veterinary care as per an existing Veterinary Client Patient Relationship (VCPR). No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required. This study conformed to the ARRIVE 2.0 guidelines of reporting (Addendum 6). Clients were offered both treatments and they could opt for one treatment (see later).

3.2 Experimental Design, Randomisation and Treatments

3.2.1 Study design

A prospective trial was performed comparing two different treatments of KCS in dogs. Client-owned dogs were used in the study, with all owners signing a consent form. The inclusion criteria were dogs of any age or breed with classic clinical signs and a history of unilateral or bilateral, immune-mediated KCS and a STT-1 $\leq$ 15 mm/min; with no co-existing ocular pathologies; and no previous history of treatment, including tear replacements or artificial tears. Exclusion criteria were dogs with bilateral end-stage KCS (advanced corneal pigmentation, fibrosis and recurrent corneal ulceration with possible corneal perforation and blindness (Dodi, 2015b)), as well as the absence of concurrent systemic disease and medications. There was not a minimum STT-1 below which patients were excluded from the trial.

3.2.2 Sample Size and Study Population

A minimum sample size of 10 KCS eyes per treatment group was calculated using a paired t-test power curve calculation (using the following assumptions: alpha = 0.05; beta = 0.20; power = 0.80; mean difference = 3.0 mm/min on STT-1 on examination 30 days after treatment; standard deviation of the differences = 3 mm/min). Sampling was opportunistic and dogs were assigned to a treatment group based on owners' willingness to participate and preference after explaining the two treatment options and costs in an unbiased standardised manner. All owners were provided with the same information so as not to bias their decision. Clients were invoiced at market related prices for each treatment and no discounting or other forms of enticement was offered to use their dogs in the present study. Because of

opportunistic sampling and owners paying for treatments, randomization of dogs to a treatment group could not be done. None of the dogs from the trial were kept in the hospital overnight.

3.2.3 Treatment Groups

A total of 2 treatment groups were used in this study, a Tacrolimus group and Stem Cells group. Dogs assigned to the Tacrolimus group were examined in the consult room and then treatment was started that same day by the owner. A standardised tacrolimus 0.02% eyedrop solution with the same oil carrier, from the same compounding pharmacy and batch was used (batch number VT014767; V-Tech; South Africa). This eyedrop solution was applied twice-daily as a single drop per eye. If the owner had not used eyedrops in their dog before, the first drops were demonstrated to them by a South African qualified veterinary ophthalmologist.

Dogs assigned to the Stem Cells group were admitted to VFAH for a day procedure and were discharged into the owner's care in the afternoon. A commercially available MSC solution (5 million viable MSC/mL; 2 mL per vial; VetRenew; South Africa) was used. VetRenew harvests dog adipose tissue from female dogs undergoing elective ovariohysterectomy after obtaining owner consent for donation. Approximately 10 grams of adipose tissue is harvested. A qualified and experienced cellular biologist breaks down the adipose tissue to harvest a stromal vascular fraction which has approximately 5% MSC. The stromal vascular fraction is then suspended in a culture medium in plastic culture bottles and placed into an incubator (37.5°C and 5% carbon dioxide; ESCO CellCulture CO2 incubator; ESCO Technologies; RSA). The cellular biologist passaged the cell culture by harvesting MSC cells and placing them in new culture bottles until a pure MSC culture grows, verified on microscopic examination (MSC demonstrate characteristic identifiable features and stick to plastic). On the 5th passage, the pure MSC culture is analysed to verify their cell surface markers using flow cytometry (CytoFlex; Beckman Coulter; Beckman Coulter Eurocenter SA; RSA). The MSC must express CD90 (CD90-APC Alexa Fluro750; Beckman Coulter) and can express < 5% CD34 (CD34-APC; Beckman Coulter) but not express CD45 (CD45-FITC; Rat Anti-dog; BioCom Africa; RSA) and MHC II (MHC Class II monomorphic-RPE; BioCom Africa; RSA). Cell viability is assessed by using a viability dye (ViaKrome 638 Fixable Viability Dye; Beckman Coulter). Every vial that is bottled is prepared with 2 mL of 5 million viable MSC/mL. Cell counts are done using the flow cytometer and a benchtop cell analyser (ProCyte, IDEXX). The MSC are suspended in phosphate buffered saline and foetal bovine serum stabilised with penicillin and streptomycin. Dogs in this group were cared for by experienced staff at VFAH for the day but were not provided food and water due to them requiring sedation to perform the peri-lacrimal gland injection of MSC (see later).

3.3 Study Procedures

Data were collected during an ophthalmic examination performed at three timepoints; before treatment which was indicated as Day 0 (0), Day 30 (30) and Day 60 (60) after starting treatment. The ophthalmic

examination included a STT-1 (Schirmer Tear Test-1, MSD Animal Health, South Africa; Schirmer Tear Test strips were all of the same lot throughout the trial period); slit lamp biomicroscopy (Keeler PSL Classic, Keeler LTD SL4 4AA, United Kingdom) to examine the cornea, anterior structures and lens; indirect ophthalmoscopy (Volk 20D Double Aspheric Binocular Indirect Ophthalmoscope, Ohio, USA) of the fundus; tonometry (Tonovet Tonometer, Type TV01, Toilat Oy, Finland) to measure intraocular pressure (IOP); close-up photographic image (photographs using a iPhone 8 cell phone camera); TBUT using fluorescein stain (Bausch & Lomb Minims Fluorescein Sodium 2% w/v, eye drops solution in 1 sterile single dose container of 0.5 mL) and a fluorescein assessment for ulcers. The TBUT was timed using a cell phone timer application; as follows: a drop of fluorescein was dropped into the eye, the patient's eye was then closed, the timer was started, the eye opened and examined through the slit lamp for the endpoint being when the fluorescein begins to break apart on the surface of the cornea. The cobalt blue setting on the same slit lamp was used to examine the TBUT and all evaluations were done by the same veterinary ophthalmologist. The corneas were also scored at initial diagnosis and each successive follow-up examination using an author-derived simple descriptive system (Table 3.1).

Dogs assigned to the Stem Cells group underwent a clinical examination, including temperature, heart rate, respiratory rate, assessment of mucous membranes and capillary refill time. A haematocrit and blood biochemistry, to measure blood urea nitrogen and creatinine were also performed. If the dog was deemed healthy following this examination, then it was sedated. A standardised sedation protocol where a combination of butorphanol (0.1 mg/kg; MSD Animal Health, South Africa) and medetomidine (0.01 mg/kg; Zoetis South Africa (Pty) Ltd) was injected by separate injections into a previously place intravenous catheter (Smiths Medical Jelco; South Africa (Pty) Ltd). Once sedated (unable to lift their head), the MSC were injected into the general area of the lacrimal gland of both eyes. If sedation was inadequate, an intravenous bolus of propofol (1 mg/kg; Fresenius Kabi; South Africa) was given. The volume of MSC injectate was weight dependant: dogs < 15 kg were injected with 0.5 mL, and dogs > 15 kg were injected with 1 mL, per eye, respectively. A 2 to 3 cm shaved margin of the dorsal eyelid was used, with povidone-iodine (Adcock Ingram, South Africa) being used as the preparation medium. Using a McPherson's forceps, the lateral third of the upper eyelid was stabilised. The MSC solution, drawn up in a 1 mL syringe (Surgi-Plus; South Africa) along with a 21-gauge needle (Healthease; South Africa) was inserted a few millimetres ventral to the lateral orbital rim, underneath the skin, towards the midline of the upper eyelid, advanced obliquely for approximately 5 mm where a third of the injectate was administered. The needle was then retracted to just beneath the skin and redirected medially, a few millimetres from the initial infusion site towards the middle of the lateral third of the upper eyelid and was advanced obliquely in the direction of the eyelid margin for roughly 5 mm. A third of the treatment was injected here. The needle was then retracted for a last time to just beneath the skin and then advanced obliquely in the direction of the lateral canthus for about 5 mm. The remaining

third of the treatment was injected here before completely retracting the needle (see Figure 3.1). The same veterinary ophthalmologist performed all stem cell implantations. This standardises the technique of the procedure but unfortunately not being blinded to the treatment groups.

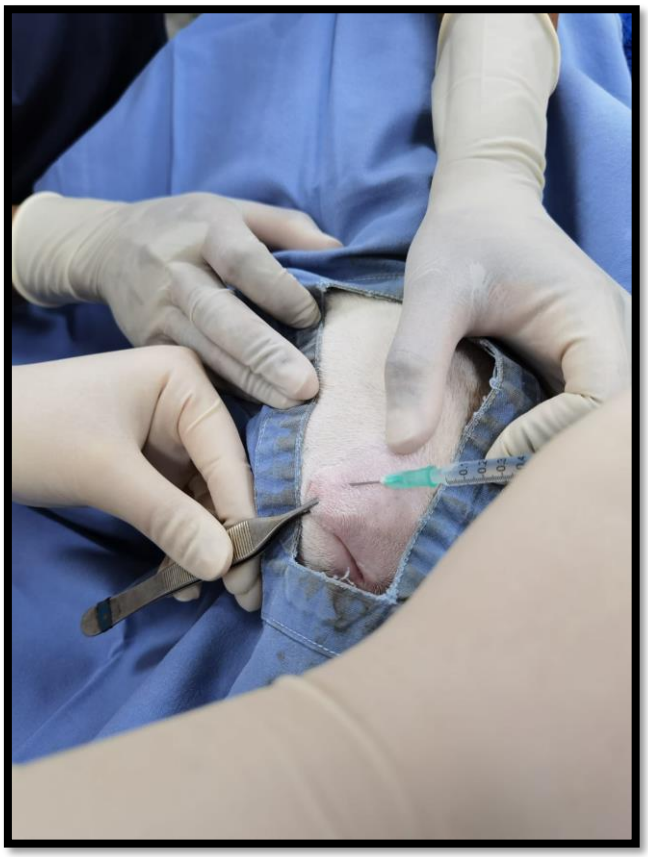
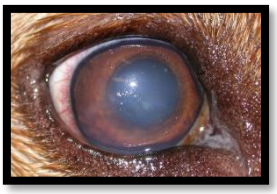


Figure 3.1 Depiction of Stem Cell procedure whereby MSC solution was drawn up into a 1mL syringe and injected obliquely in the direction of the lateral canthus whereby the remaining third of the treatment was injected before retracting the needle.

Table 3.1 Author-derived simple descriptive corneal scoring system.

0	Clear cornea, uniform tear film (using slit lamp and fluorescein).	
1	Clear cornea, lusterless, uneven surface (using slit lamp and fluorescein), associated mucus.	
2	Mild corneal hazing/corneal scarring/(no vascularization), uneven surface, associated mucus.	
3	Early superficial vascularization, corneal hazing, uneven corneal surface, associated mucus.	
4	Establishing superficial keratitis, uneven corneal surface, associated mucus.	
5	Established keratitis with pigmentation, uneven corneal surface and associated mucus.	

The sedative effects of medetomidine were antagonised using a single intramuscular injection of atipamezole (Zoetis, South Africa) administered at 5 times the dose of medetomidine. Dogs were monitored for any signs of hypersensitivity (cutaneous wheals, hypotension, tachycardia, vomiting, diarrhoea, itchiness) or untoward effects of injection (heat, swelling and redness) after administration of the injectate until they were fully awake from the sedation. Once fully awake and recovered, they were discharged on the same day as the procedure was performed into the care of their owners.

All dogs received topical lubricating eyedrops (Tears Naturale; Alcon® Laboratories Inc, South Africa) at two to three times daily for the duration of the study. This ensured palliative and symptomatic relief from the KCS condition. The same brand and formulation were used for all dogs. The owner was asked to not administer these lubricating eyedrops on the data collection days.

3.4 Rescue interventions

If dogs in the Stem Cells group showed signs of hypersensitivity to injection of MSC, blepharospasm or scratching at the eye, then an opioid (morphine 0.3 mg/kg IM; Fresenius Kabi, South Africa) and/or non-steroidal anti-inflammatory drug (meloxicam 0.2 mg/kg SC; Ascendis Animal Health (Pty) Ltd, South Africa) was administered.

3.5 Data collection and data analysis

Data collected during the ophthalmic examination were recorded on separate data capture sheets for both eyes for each dog for all three data captures (0, 30, 60). At each data collection, the owner was asked if they had any comments on the treatment plan. Data from each eye was collected for the following: STT-1, TBUT, IOP and corneal score.

Once completed, data was uploaded to spreadsheets (Microsoft Excel). A third investigator, masked to the treatments was given the datasheet for analysis. Then each eye was evaluated to classify them as healthy (STT-1 > 15 mm/min) or KCS (STT-1 ≤ 15 mm/min). The distribution of the data was evaluated by inspecting descriptive data, histograms and applying the Anderson-Darling test for normality. Data were normally distributed and therefore reported as mean (95% confidence interval of the mean) or mean (minimum, maximum). For hypothesis testing, a mixed effect model (fixed effect: time, treatment; random effect: dog, eye) was used to compare STT-1, TBUT and corneal score values using the following interactions: treatment, time, and treatment x time (Brown and Prescott, 2015, Beitler and Landis, 1985) between treatments within healthy eye and KCS eye classifications, respectively. For non-hypothesis data analyses, the same mixed effect model was used to compare IOP between treatments within healthy eye and KCS eye classifications, respectively. Significant interactions were compared using post-*hoc* Bonferroni pairwise comparisons. Significance was interpreted at a P-value of less than 0.05. Commercial software was used for statistical analysis (MiniTab 18.1; MiniTab Inc.; USA).

Chapter 4 Results

A total of 22 dogs (44 eyes) were examined and were of the following breeds: Yorkshire Terriers (Stem Cells: 2; Tacrolimus: 3), Standard and/or Giant Schnauzers (Stem Cells: 2; Tacrolimus: 2), Bullterriers (Stem Cells 1; Tacrolimus: 1), Boerboel (Tacrolimus: 1), Dachshunds (Stem Cells: 1; Tacrolimus: 1), West Highland White Terrier (Tacrolimus: 1), Pekingese (Stem Cells: 1; Tacrolimus: 1), Cavalier King Charles Spaniel (Tacrolimus: 1), Wire-haired Fox Terrier (Tacrolimus: 1), English Bulldog (Stem Cells: 1), Cane Corso (Stem Cells: 1) and Afghan (Stem Cells: 1). The age of the dogs was a mean (min; max) of 8.7 (1.5; 14.0) years old with no difference between treatment groups. Of the 22 dogs used, 1 from Stem Cells and 2 from Tacrolimus were excluded for not meeting the STT-1 inclusion criteria at Day 0, and 2 were excluded from the Tacrolimus group because they were lost during the Day 30 and Day 60 data collection period. Thus, a total of 9 dogs were used in Stem Cells (4 healthy eyes and 14 KCS eyes) and 8 dogs in Tacrolimus (4 healthy eyes and 12 KCS eyes) were suitable for data analysis.

4.1 Schirmer Tear Test

The STT-1 at Day 0 in healthy eyes were 20 (16, 23) and 21 (11, 31) mm/min for Stem Cells and Tacrolimus, respectively and not significantly different at Day 30 (Stem Cells: 18 [8, 28] mm/min; Tacrolimus: 26 [16, 36]; both treatment x time interaction P = 0.253) and Day 60 (Stem Cells: 22 [16, 27] mm/min; Tacrolimus: 27 [20, 34]; Figure 4.1.1 and Table 4.1.1). The STT-1 at Day 0 in KCS eyes were 11 (9, 13) and 11 (8, 14) mm/min for Stem Cells and Tacrolimus, respectively. The STT-1 increased in both treatment groups over time and measured as 18 (16, 21) and 19 (15, 22) mm/min for Stem Cells and Tacrolimus at Day 30, respectively (both P < 0.001). The STT-1 stabilised in the KCS eyes at Day 60 were 18 (15, 22) and 19 (15, 23) mm/min for Stem Cells and Tacrolimus, respectively (both P < 0.001 pairwise to 0 days). The ranges of STT-1 for each treatment within healthy eyes and KCS eyes are tabulated in Table 4.1.1.

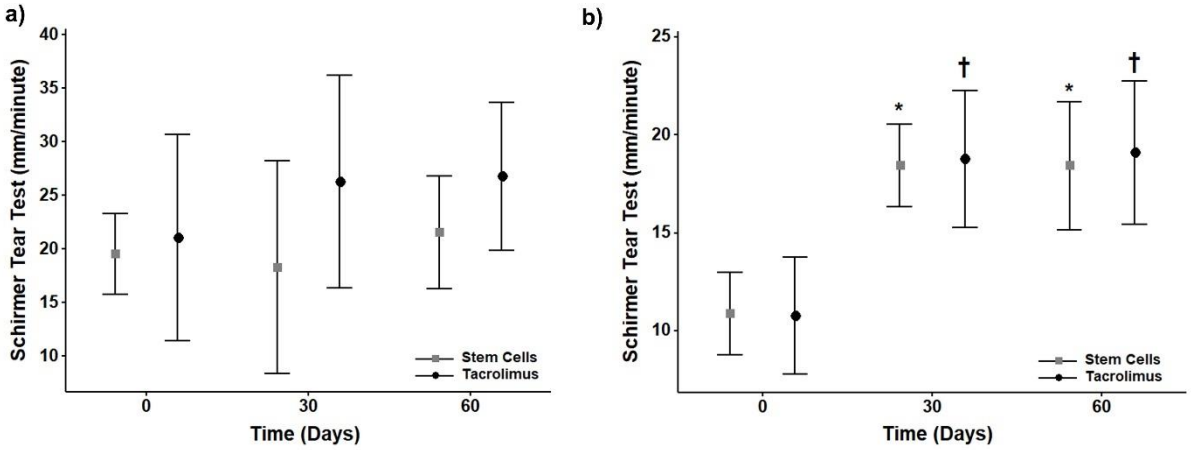


Figure 4.1.1 Mean (95% confidence interval of the mean) values for Schirmer Tear Test (STT-1: mm/min) measured before treatment (0) and at Day 30 (30) and Day 60 (60) after treatment in 17 dogs with either unilateral or bilateral presumed KCS. The eyes were classified as either healthy eye (STT-1 > 15 mm/min) (a) or KCS eyes (STT-1 ≤ 15 mm/min) (b). Dogs underwent either once off Stem Cells treatment (*n* = 9 dogs) or twice-daily Tacrolimus treatment (*n* = 8 dogs). * and † indicate pairwise significant differences compared to Day 0 over time for Stem Cells and Tacrolimus, respectively.

Table 4.1.1 The mean, minimum and maximum values for STT-1 (mm/min) measured before treatment (0), and at Day 30 (30) and at Day 60 (60) collected in 17 dogs with either unilateral or bilateral presumed KCS. Eyes were either classified as healthy (STT-1 > 15mm/min) (Stem cells and Tacrolimus $n = 4$) or KCS eyes (STT-1 ≤ 15mm/min) (Stem cells $n = 14$ and Tacrolimus $n = 12$)

Time (Days)	Mean (mm/min)	Minimum (mm/min)	Maximum (mm/min)
		Stem Cells group	
		Healthy eyes ($n = 4$ eyes)	
0	20	18	23
30	18	10	25
60	22	17	24
		KCS eyes ($n = 14$ eyes)	
0	11	5	15
30	18	10	25
60	18	8	27
		Tacrolimus group	
		Healthy eyes ($n = 4$ eyes)	
0	21	17	30
30	26	20	33
60	27	23	31
		KCS eyes ($n = 12$ eyes)	
0	10	4	15
30	19	9	28
60	19	8	26

4.2 Tear Break-up Time

The TBUT at Day 0 in healthy eyes were 22 (12, 31) and 17 (3, 31) seconds for Stem Cells and Tacrolimus, respectively and different at Day 30 (Stem Cells: 25 [9, 40] seconds; Tacrolimus: 34 [7, 60]; both treatment x time interaction P = 0.001) and Day 60 (Stem Cells: 29 [15, 42] seconds; Tacrolimus: 23 [0, 46] seconds; Figure 4.2.1 and Table 4.2.1). The TBUT at Day 0 in KCS eyes were 21 (11, 32) and 18 (12, 25) seconds for Stem Cells and Tacrolimus, respectively. The TBUT increased in both treatment groups over time and measured as 23 (16, 30) and 27 (21, 33) seconds for Stem Cells and Tacrolimus at Day 30, respectively (both P < 0.001) and at Day 60 were 36 (27, 44) and 30 (22, 39) seconds for Stem Cells and Tacrolimus, respectively (both P < 0.001). The ranges of TBUT for each treatment within healthy eyes and KCS eyes are tabulated in Table 4.2.1.

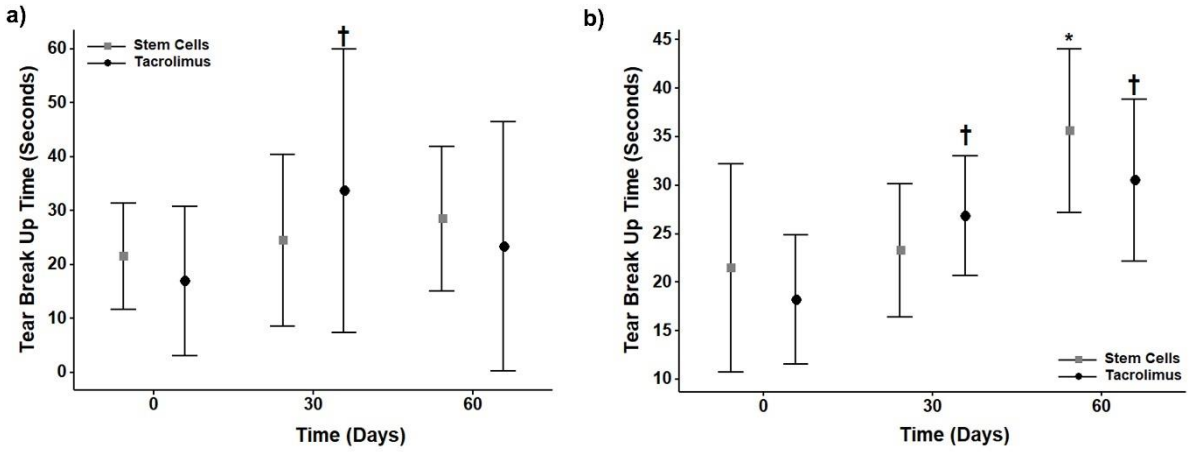


Figure 4.2.1 Mean (95% confidence interval of the mean) values for Tear Break Up Time (TBUT: seconds) measured before treatment (0) and at Day 30 (30) and Day 60 (60) after treatment in 17 dogs with either unilateral or bilateral presumed KCS. The eyes were classified as either healthy eye (STT-1 > 15 mm/min) (a) or KCS eyes (STT-1 ≤ 15 mm/min) (b). Dogs underwent either once off Stem Cells treatment (*n* = 9 dogs) or twice-daily Tacrolimus treatment (*n* = 8 dogs). * and † indicate pairwise significant differences compared to Day 0 and Day 30 for Stem Cells and Day 0 for Tacrolimus, respectively.

Table 4.2.1 The mean, minimum and maximum values for TBUT (seconds) measured before treatment (0), and at Day 30 (30) and at Day 60 (60) collected in 17 dogs with either unilateral or bilateral presumed KCS. Eyes were either classified as healthy (STT-1 > 15mm/min) (Stem cells and Tacrolimus *n* = 4) or KCS eyes (STT-1 ≤ 15mm/min) (Stem cells *n* = 14 eyes and Tacrolimus *n* = 12 eyes). Dogs underwent either once off Stem Cells treatment (*n* = 9 dogs) or twice-daily Tacrolimus treatment (*n* = 8 dogs).

Time (Days)	Mean (seconds)	Minimum (seconds)	Maximum (seconds)
		Stem Cells group	
		Healthy eyes (*n* = 4 eyes)	
0	22	16	30
30	15	17	39
60	29	23	41
		KCS eyes (*n* = 14 eyes)	
0	21	8	50
30	23	9	52
60	36	16	53
		Tacrolimus group	
		Healthy eyes (*n* = 4 eyes)	
0	17	11	22
30	34	24	45
60	23	13	31
		KCS eyes (*n* = 12 eyes)	
0	18	8	36
30	27	15	41
60	30	13	45

4.3 Intraocular Pressure

The IOP at Day 0 in healthy eyes were 13 (7, 18) and 16 (6, 26) mmHg for Stem Cells and Tacrolimus, respectively and not different at Day 30 (Stem Cells: 15 [11, 19] mmHg; Tacrolimus: 15 [8, 22]; treatment x time interaction P = 0.177) and Day 60 (Stem Cells: 13 [7, 19] mmHg; Tacrolimus: 17 [10, 23] mmHg). The IOP at Day 0 in KCS eyes were 12 (10, 14) and 16 (11, 20) mmHg for Stem Cells and Tacrolimus, respectively. The IOP were similar in both treatment groups over time and measured as 14 (12, 15) and 14 (10, 18) mmHg at Day 30, respectively (treatment x time interaction P = 0.197) and at Day 60 were 14 (11, 16) and 13 (9, 16) mmHg for Stem Cells and Tacrolimus, respectively.

4.4 Corneal Scoring

The corneal scores at Day 0 in healthy eyes were 1 (0, 2) and 2 (1, 4) for Stem Cells and Tacrolimus, respectively and different at Day 30 (Stem Cells: 0 [0, 1]; Tacrolimus: 1 [0, 3]; both time interaction P < 0.001) and Day 60 (Stem Cells: 0 [0, 0]; Tacrolimus: 1 [0, 3]; both time interaction P = 0.003). The corneal score at Day 0 in KCS eyes were 2 (1, 3) and 2 (1, 3) for Stem Cells and Tacrolimus, respectively. The corneal scores decreased in both treatment groups over time and measured as 1 (0, 2) and 1 (0, 2) for Stem Cells and Tacrolimus at Day 30, respectively (both time interaction P < 0.001). The corneal scores remained relatively constant at Day 60 and were 1 (0, 2) and 1 (0, 2) for Stem Cells and Tacrolimus, respectively (both time interactions P < 0.001 pairwise to Day 0).

4.5 Patient 8 from the Stem Cells Group

One of the patients in the Stem Cells group underwent treatment and suffered a mild unilateral reaction thereafter with chemosis of the conjunctiva of the right globe. In a study conducted by (Bittencourt et al., 2016) in Brazil, in which allogeneic mesenchymal stem cells were transplanted in dogs suffering from KCS as in the present study, it was noted that no side effects such as epiphora, inflammation, photophobia, ocular pain, blinking or blepharospasm, were noted with the patients' eyes following mesenchymal stem cell transplantation in the short term (7-28 days), or the long term (6-12 months) (Bittencourt et al., 2016). There were also no changes reported amongst any of the patients with regards to faecal output, appetite, body temperature, or weight, and importantly, no allergic reactions were noticed (Bittencourt et al., 2016). This particular patient in the study received the same allogeneic mesenchymal stem cells as all other patients in the trial. Both eyes were surgically prepped in the same manner, with the same amount of the medium having been injected peri-lacrimally, but the patient still maintained only a unilateral reaction. The conjunctival chemosis however, responded well to being treated with antihistamines, with the swelling subsiding over the course of the afternoon. 4 days post-procedure in the same dog, she developed a swollen upper eyelid on the same affected side as the day of the procedure, which responded well to Exocin twice-daily and Metacam per os once daily. The swelling had subsided after a re-check 2 days later.

4.6 General Owner Comments

Most owners in Tacrolimus group reported challenges around the administration of the eyedrops to their dogs which lead to issues on compliance. Despite these challenges the owners were satisfied that the STT-1 and TBUT improved. All owners with dogs in the Stem Cells group only had positive comments. Owner comments are summarised in Table 4.7.1.

Table 4.6.1: General owner comments and remarks throughout the trial process at various follow-ups

Patient Number	Follow-up Day (30-day or 60-day)	Owner comment/remark
	Tacrolimus Group	
1	30	Owner battles to do 1 drop BID; been inconsistent. Some days are missed and other days only gives one drop
1	60	Owner remarked on needing to find an alternative way to apply the drops. She is unable to see if they are coming out of the bottle, landing in the eye or somewhere on the face
2	30	Owner remarked that she struggles to get the drops in as the dog won't sit still when trying to put them in
2	60	No drops were being given at this stage. The owner noted that the dog is non-compliant and moves too much
3	30	Owner noted that he had been managing well with the drops twice-daily into both eyes consistently
5	60	Owner noted that she stopped giving the drops +- 3 weeks before the third follow-up as the dog became averse to treatment
11	60	Owner noted that some days the Tacrolimus drops were missed or only given once daily
	Stem Cells Group	
3	60	Owner reported significantly less mucous across both eyes
4	60	Owner noted that eyes appear much clearer in appearance and are producing much less mucous overall
7	60	Owner remarked that the dog rubs his face much less; the sclera is much less red in appearance and the dog overall seems much happier and more comfortable
8	30	Owner commented that the dog is scratching/cleaning her face much less
8	30	Owner noted that they are generally happy with how the patient's eyes are looking and with how she is doing after the procedure
10	60	Owners remarked that they are very happy with how the dog's eyes are looking after the procedure; that he appears to be much more comfortable and is producing less mucous than before

Chapter 5 Discussion

The results of this study show that the use of AD-MSC may be as effective in the treatment of canine KCS as tacrolimus. All dogs' values with respect to STT-1 and TBUT normalised to within reference intervals over the course of the trial period and Corneal Scoring improved while IOP remained within reference intervals.

While stand-alone trials, concerning the use of both tacrolimus and stem cells as a treatment for immune-mediated canine KCS have been reported, there has not been, to the best of our knowledge, a comparative study of these therapeutic options. One study determining the efficacy of allogeneic MSC transplantation; with a minor difference of additionally injecting MSC's into the lacrimal gland of the third eyelid; proved beneficial and 'improved over time' (Bittencourt et al., 2016). However, while the study proved successful, it was not comparative. In another study in which mesenchymal stem cells were used topically via conjunctival administration, it was shown that treated animals produced a significant reduction in inflammatory markers such as CD4, IL-6, IL-1 and TNF-α (Sgrignoli et al., 2019). However, this study concluded that the stem cell topical medication would need to be used as adjuvant therapy, along with a topical immunosuppressive agent such as tacrolimus, cyclosporine or pimecrolimus, in the treatment of the KCS condition in dogs (Sgrignoli et al., 2019). Another study in which mesenchymal stem cells were administered intravenously to patients suffering from the KCS condition proved to be more successful when the anatomical changes to the lacrimal gland itself were less profound (Hermida-Prieto et al., 2021); when the disease had not yet progressed too far and still had the opportunity for reversion (Hermida-Prieto et al., 2021). Consequently, while all studies prove the efficacy of stem cell use, the less localised the placement of the stem cell medium around the lacrimal gland, the less profound the outcome. Tacrolimus eyedrops, in various suspensions have been trialled and tested against other immunosuppressive topical drugs such as cyclosporine and pimecrolimus. It was proven that twice-daily administration of 0.02% tacrolimus in this case, aqueous suspension, adequately increases tear production in dogs and proves to be an encouraging substitute to topical cyclosporine, especially in patients who show a less than favourable response to topical cyclosporine drops (Berdoulay et al., 2005). Tacrolimus eyedrops diluted in linseed oil and olive oil were both efficacious in the treatment of KCS (Zulim et al., 2018). The linseed oil based drop did, however, show significantly lower neutrophil counts, so was considered as a more favourable alternative diluent than the olive oil (Zulim et al., 2018). Pimecrolimus at a concentration of 1% proved to be highly efficacious in abating the clinical signs in dogs suffering from KCS with respect to ocular surface inflammation as well as tear secretion (Nell et al., 2005). It demonstrated superiority over cyclosporine in the control of certain clinical signs involving corneal and conjunctival inflammation after 8 weeks of treatment (Ofri et al., 2009). Topical immunosuppressive drugs have therefore proven their efficacy in multiple trials with tacrolimus being the most productive and effective in abating clinical signs. Conclusively, both topical oil-based tacrolimus eyedrops and a peri-lacrimal gland

injection of AD-MSC's prove to be beneficial and effective in the treatment of the KCS condition. The results of the present study prove that the two therapeutic modalities are effective. It is important to note that when comparing tacrolimus against stem cells, owing to the direct lacrimostimulant of tacrolimus (Barabino et al., 2020), The STT-1 values increased in the 'healthy eyes' from values of 21 to 26 then 27 mm/min. This effect however, was not seen within the stem cells group with STT-1 values beginning at 20, then decreasing to 18 and the fluctuating to 22 mm/min.

Certain drugs and combinations thereof may also play a role in predisposing to decreased tear production. In our study, a combination of butorphanol and medetomidine was used for sedation. (Leonardi et al., 2019) reported that medetomidine, butorphanol, as well as combinations of these drugs reduce tear production. The most likely explanation of this quantitative decline in tear production is due to evaporative loss as a result of the sedative effects of this drug combination, which tends to cause less blinking (Bufalari and Lachin, 2015), resulting in the aqueous layer having more ability to evaporate. The decreased tear production was not a concern for the study owing to the dogs being previously diagnosed with KCS before being used in the study. Furthermore, the risk of the medetomidine-butorphanol combination used for sedation to worsen the KCS over time was minimal.

Verbal feedback from dog owners were noted throughout the trial and although subjective, Tacrolimus group owners described challenges around dog and owner compliance as compared to owners with dogs in the Stem Cells group. These owners noted that they are unable to get the eyedrops in; require many people to hold the patient in order to get them in or only manage once a day or every other day applications. Whereas, owners of the dogs in the Stem Cells group had positive feedback, noting that their dogs appear much more comfortable than before; that they scratch their face less, have much less ocular discharge and overall appear to be much happier in themselves. The Stem Cells procedure is one-time; however, it is not yet considered a mainstay treatment in most veterinary practices perhaps because of a previous scarce availability in many countries. The tacrolimus eyedrops are known to be effective in treating the KCS condition, provided the owners can commit to twice-daily, lifelong treatment administration. This proved challenging for many of the owners involved within this trial. In South Africa, the oil-based Tacrolimus eyedrops cost approximately R500 while in this trial the cost of the stem cells procedure was approximately R16 000. One bottle of the oil-based tacrolimus eyedrops lasts around 1 months' duration. While immune-mediated KCS is a life-long condition, affected animals require life-long treatment. Bittencourt et al. (2016) noted that STT-1's were recorded in patients up until one year later subsequent to stem cell treatment and still maintained STT-1's within adequate tear production ranges. The stem cell treatment, as demonstrated in dog osteoarthritis studies (Kriston-Pál et al., 2020; Yoon et al., 2012), may prove to be effective over a longer period of time (five years or longer) and therefore may too, result in being a more cost-effective therapy in the long-term.

The study was not without limitations, the first being the author-derived corneal scoring system which allowed for bias. Since it is author-derived, it did create room for subjectivity and on reflection perhaps there were too many possible categories proposed in this system. However, the many categories were by design to try and identify subtle changes in corneal health. Another limitation to this scoring system is that it is not validated but it did provide a tool to monitor subtle changes in corneal healthy in these dogs. With there not being randomised selection between the treatment groups was another means in which bias was created within the study as well as the fact that investigators collecting the data were not masked to the treatment groups. However, to minimise bias, the third investigator was handed a blinded datasheet for analysis, therefore blinding occurred at the data analysis stage of the study. In order to include dogs in the early stage of the disease, all of them had to have the classic clinical ocular signs and an owner history of the dog rubbing their face. An experienced South African qualified veterinary ophthalmologist selected the dogs that she would have started treatment, regardless of the study, based on clinical signs but taking the STT into considerations, as previously described (Herrera, 2005). Therefore, dogs with mild disease, early-stage KCS were included and both eyes were treated even if KCS was only unilateral (n = 4 in both groups). The owners consented to treating both healthy and KCS eyes in the dogs with unilateral disease. The tear production improved in these healthy eyes over time which provided important and useful information that, to the authors knowledge, has never been reported. This information can be used to perform accurate sample size calculations for future randomised controlled studies. The authors of this dissertation do not believe that any harm was caused to these dogs with unilateral KCS. From a clinical perspective, the authors recommend to only treat dogs with eyes that are diagnosed having KCS which could present as unilateral or bilateral disease. Furthermore, we cannot speculate if these treatments would have had the same outcome effect if given to dogs with a more advanced stage of KCS.

Chapter 6 Conclusion and Critical Evaluation

6.1 Conclusion

To conclude, a single peri-lacrimal gland injection of adipose-derived mesenchymal stem cells is effective to increase tear production (indicated by STT-1) and tear film stability (indicated by TBUT) and was similar to twice-daily application of tacrolimus eyedrops over a 60-day period in dogs with immune-mediated keratoconjunctivitis sicca. These results should be interpreted with a caveat, dogs with early signs of KCS were investigated but we cannot comment on what the effect would be in dogs with more advanced KCS. Furthermore, we only followed the dogs for 6 months and cannot comment on how long the duration of effect would be when treating KCS with mesenchymal stem cells.

6.2 Critical Evaluation

This study was performed to evaluate and compare the efficacy of a one-time peri-lacrimal gland injection of AD-MSC to that of twice-daily use of tacrolimus eyedrops over a 60-day period. All patients within the Stem Cells group underwent the same procedure previously described, while all patients in the Tacrolimus group received eyedrops from the same compounded batch.

Owing to the corneal scoring system being one that is author-derived, it did create room for subjectivity and on reflection perhaps there were too many possible categories proposed in this system. Another limitation to this scoring system is that it is not validated but it did provide a tool to monitor a change in corneal healthy in the dogs. Another limitation is that dogs with early-stage KCS were enrolled. Some of the dogs only had unilateral STT-1 < 15 mm/min (n = 4 in each group). Therefore, our results have only demonstrated similar benefits in these dogs, and we cannot speculate if the same outcome would be true in dogs with more severe KCS. Perhaps another trial can be conducted whereby all patients are considered end-stage and will therefore determine the efficacy of the MSC.

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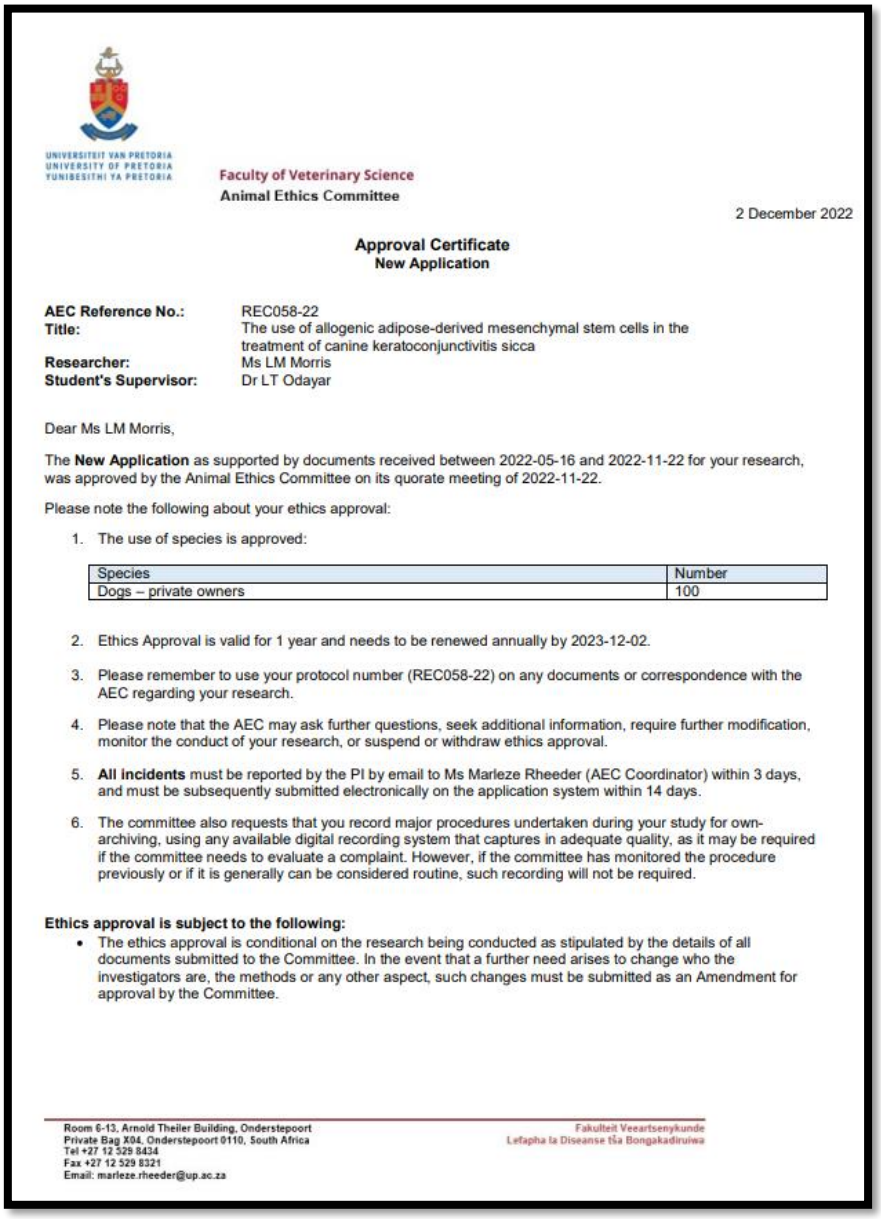
Addendums

Addendum 1 AEC Certificate



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

2 December 2022

Approval Certificate
New Application

AEC Reference No.: REC058-22
Title: The use of allogenic adipose-derived mesenchymal stem cells in the treatment of canine keratoconjunctivitis sicca
Researcher: Ms LM Morris
Student's Supervisor: Dr LT Odayar

Dear Ms LM Morris,

The **New Application** as supported by documents received between 2022-05-16 and 2022-11-22 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-11-22.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Dogs – private owners	100

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-12-02.
3. Please remember to use your protocol number (REC058-22) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.

Yours sincerely

Prof A Tordiffe
DEPUTY CHAIRMAN: UP-Animal Ethics Committee

Addendum 2 Owner Consent Form

1

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INFORMED CONSENT

Owner:
Owners ID:
NB: Circle Primary Contact:
Cell 1
Cell 2
E-mail
Dog sitter -

Patient:
Breed: **Age:** **Sex:** **Weight:** **Color:**
Hospitalising Vet:

The use of allogenic adipose-derived mesenchymal stem cells in the treatment of canine keratoconjunctivitis sicca

The following research project has passed the Research and Ethics committees of the University of Pretoria, Faculty of Veterinary Science, with the following Reference Number:

Should you have any questions related to this project or procedure then please contact the head of the research project Dr Laurie Morris directly on: 082 559 1940

Please note that this facility namely, **Valley Farm Animal Hospital** is merely providing the premises and professional staff to conduct the treatment and is not responsible for this research project.

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2

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Keratoconjunctivitis sicca (KCS), more generally known as dry eye, is an inflammatory condition of the ocular surface. The precorneal tear film (PTF) is constituted of 3 components that make contact with both the cornea and conjunctiva and it essentially lubricates the eyeball. KCS is a condition whereby certain components of the PTF becomes deficient, which is why the condition is commonly given the name 'dry eye'. There are many proposed causes of this condition, but the most commonly proposed cause is said to be immune-mediated. Owing to the drying out of the ocular surface, patients often present with reluctancy to open their eyelids due to pain, a mucoid-like ocular discharge, redness of the surrounding ocular tissues and in severe cases, secondary bacterial infections (Dodi, 2015). It's therefore not a condition that can be left alone, it always requires medical intervention.

Basic diagnostics of the condition require Schirmer Tear Test (STT) and Tear Break-Up Time (TBUT) values to come to a conclusion. Both tests are a quantitative measure of tear production. With respect to STT values, >15mm/minute is considered normal, 6-10mm/minute is moderate and 5mm/minute or less is extremely deficient. With respect to TBUT, normal values range between 15 and 25 seconds while values of 10 seconds or less indicate a reduction in tear film (Lewin, 2014).

Tacrolimus has been the mainstay treatment of choice for patients suffering from KCS for many years. Previous to that, cyclosporine was utilised, both due to having immune-suppressant properties. Tacrolimus, however, is an eyedrop requiring application twice a day, with the treatment time being extensive.

Mesenchymal stem cells (MSC) have proven to possess massive regenerative properties when used in other veterinary fields with great success. The trial will investigate MSC's potential as a once-off treatment as a therapy for KCS. The patients will be placed under sedation and the MSC's injected into the lacrimal gland.

Two follow-up examinations will be required, 30- and 60- days following treatment initiation, to gather data and

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3

Valley Farm Animal Hospital
BECAUSE YOUR PET'S HEALTH MATTERS.
www.valleyfarmvet.co.za
CONSULTATIONS BY APPOINTMENT ONLY. SCAN QR CODE TO BOOK
24-Hour Emergency and Specialist Referral Practice
Principals: Drs Tim Krafft BVSc and Dean de Kock BVSc

monitor progression of the patients' condition.

DODI, P. L. 2015. Immune-mediated keratoconjunctivitis sicca in dogs: current perspectives on management. *Veterinary Medicine: Research and Reports*, 6, 341.

LEWIN, A. 2014. Keratoconjunctivitis sicca in dogs: causes, diagnosis and treatment. *Vet Times*, 6, 16-17.

General Information and Background

If having agreed to participate in the following study, and with a treatment group having been chosen, your pet will either be started on Tacrolimus eyedrops or a day will be scheduled for your pet to be admitted to the clinic for the mesenchymal stem cell treatment.

All pets will have a general clinical examination performed, involving checking all vitals and parameters, as well as a comprehensive ocular examination of both eyes.

Those pets involved in the stem cell treatment group will have bloods run as your pet will be undergoing heavy sedation. This will be done to ensure no abnormalities are detected. An intravenous catheter will be placed, and sedative drugs injected. The procedure will be performed by a veterinarian and your dog monitored until awake from the given sedation.

Post-operative pain and swelling is a potential complication following the procedure. Therefore, non-steroidal anti-inflammatories will be administered to provide for them at home.

Please be aware that in any instance involving the administration of medication, the potential of anaphylactic reactions can potentially occur. However, medication is always readily available in the case of an emergency

Follow-up examinations are a requirement for the study, which will be at 30- and 60- days following the initiation of treatment. These will be done to gather data, monitor progression of patients' condition and monitor the efficacy of the two treatment groups at hand.

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TEAMWORK · PROFESSIONALISM · RESPECT · COMMITMENT

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KCS	Yes - bilateral
STARVED	
CLIENT CONTACTED P/O	
DISCHARGE TIME	
OTHERS:	

MSC eyelid Treatment: Admission to hospital, intravenous sedation and MSC injection treatment into the dorsal eyelids.

Estimate of costs for treatment: Follow-up consultations and Tacrolimus bottles

**Please note: We always endeavour to keep costs within the estimate. However, costs are dependent on many variables, including the progression of the disease. It is sometimes impossible to predict these factors at the time of admission. If you are not available and the circumstances require an immediate decision, then treatment will be continued in the best interests of the patient and costs charged to your account. Estimates do not include follow-up consultations and treatments.*

1. I, the undersigned, an adult major, hereby authorize the veterinarians and staff of this veterinary facility to perform any reasonable treatment/sedation/anesthesia and surgery they may deem necessary, including further or alternative measures as may be necessary during the course of the surgery and/or treatment of my animal.
2. I acknowledge that a quote cannot be provided, and that I have been provided with an **estimate** for an average procedure, but the **final cost may vary** substantially and be significantly higher than discussed because of the particular factors that may be encountered as the procedure unfolds. I agree to settle the final bill in full upon request or discharge, whichever is earlier.
3. I undertake to keep in daily contact to enable the staff to inform me of progress, costs incurred and additional treatment involved.
4. I acknowledge that I am indebted to the above practice for veterinary treatment, service rendered and expenses incurred therewith and hereby render myself responsible for all costs, telephone calls and legal expenses, as between attorney and own client including collection charges that may be incurred in the recovery of the outstanding amount.

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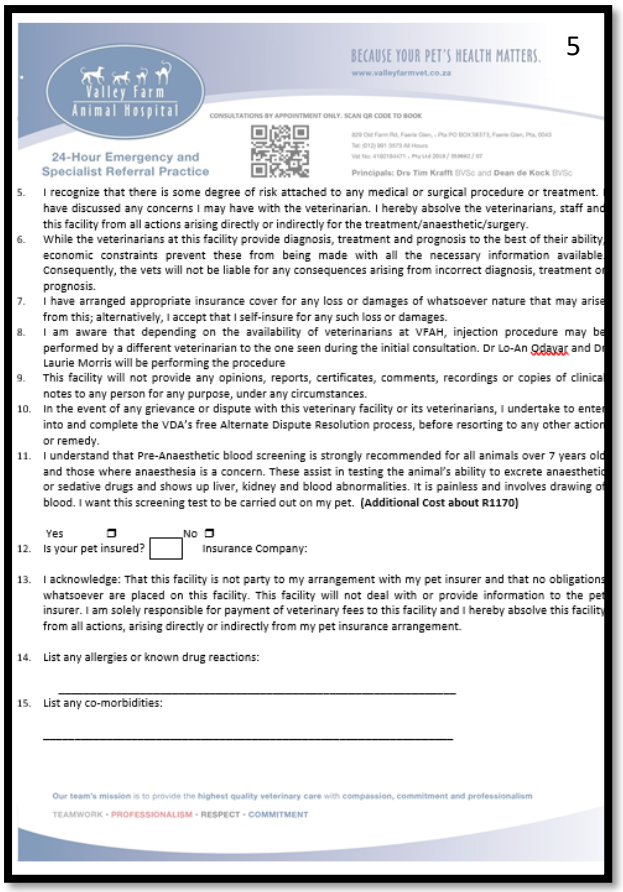

5

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CONSULTATIONS BY APPOINTMENT ONLY. SCAN QR CODE TO BOOK

829 Old Farm Rd, Faerie Glen, - Pta PO BOX38373, Faerie Glen, Pta, 0043
Tel: (012) 991 3573 All Hours
Vet No: 4160184471 - Pty Ltd 2018 / 359662 / 07

24-Hour Emergency and Specialist Referral Practice

Principals: Drs Tim Krafft BVSc and Dean de Kock BVSc

5. I recognize that there is some degree of risk attached to any medical or surgical procedure or treatment. I have discussed any concerns I may have with the veterinarian. I hereby absolve the veterinarians, staff and this facility from all actions arising directly or indirectly for the treatment/anaesthetic/surgery.
6. While the veterinarians at this facility provide diagnosis, treatment and prognosis to the best of their ability, economic constraints prevent these from being made with all the necessary information available. Consequently, the vets will not be liable for any consequences arising from incorrect diagnosis, treatment or prognosis.
7. I have arranged appropriate insurance cover for any loss or damages of whatsoever nature that may arise from this; alternatively, I accept that I self-insure for any such loss or damages.
8. I am aware that depending on the availability of veterinarians at VFAH, injection procedure may be performed by a different veterinarian to the one seen during the initial consultation. Dr Lo-An Odayar and Dr Laurie Morris will be performing the procedure
9. This facility will not provide any opinions, reports, certificates, comments, recordings or copies of clinical notes to any person for any purpose, under any circumstances.
10. In the event of any grievance or dispute with this veterinary facility or its veterinarians, I undertake to enter into and complete the VDA's free Alternate Dispute Resolution process, before resorting to any other action or remedy.
11. I understand that Pre-Anaesthetic blood screening is strongly recommended for all animals over 7 years old and those where anaesthesia is a concern. These assist in testing the animal's ability to excrete anaesthetic or sedative drugs and shows up liver, kidney and blood abnormalities. It is painless and involves drawing of blood. I want this screening test to be carried out on my pet. **(Additional Cost about R1170)**

12. Is your pet insured? Yes ☐ No ☐ Insurance Company:

13. I acknowledge: That this facility is not party to my arrangement with my pet insurer and that no obligations whatsoever are placed on this facility. This facility will not deal with or provide information to the pet insurer. I am solely responsible for payment of veterinary fees to this facility and I hereby absolve this facility from all actions, arising directly or indirectly from my pet insurance arrangement.

14. List any allergies or known drug reactions:

_____

15. List any co-morbidities:

_____

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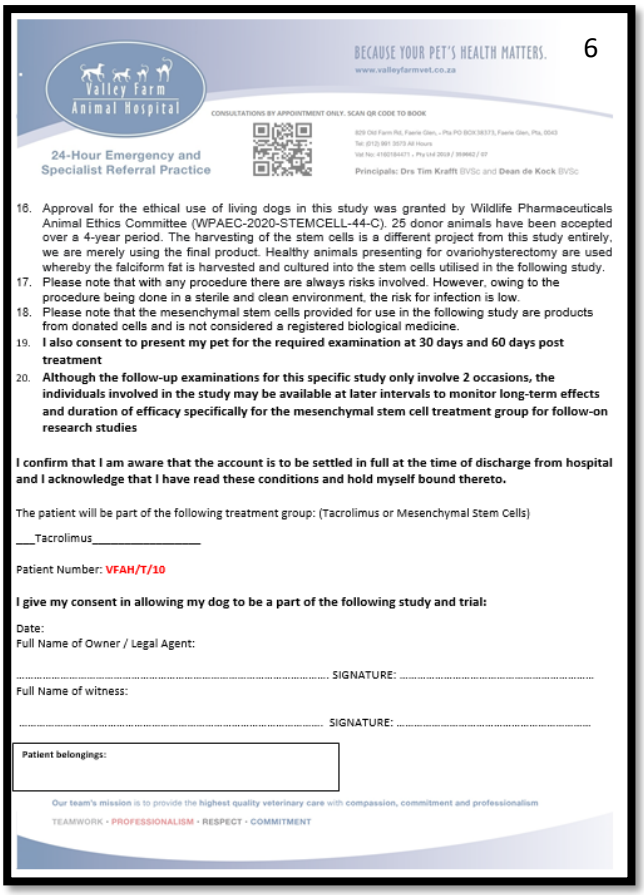

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Valley Farm Animal Hospital

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Tel: (012) 991 3573 All Hours
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24-Hour Emergency and Specialist Referral Practice

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16. Approval for the ethical use of living dogs in this study was granted by Wildlife Pharmaceuticals Animal Ethics Committee (WPAEC-2020-STEMCELL-44-C). 25 donor animals have been accepted over a 4-year period. The harvesting of the stem cells is a different project from this study entirely, we are merely using the final product. Healthy animals presenting for ovariohysterectomy are used whereby the falciform fat is harvested and cultured into the stem cells utilised in the following study.
17. Please note that with any procedure there are always risks involved. However, owing to the procedure being done in a sterile and clean environment, the risk for infection is low.
18. Please note that the mesenchymal stem cells provided for use in the following study are products from donated cells and is not considered a registered biological medicine.
19. **I also consent to present my pet for the required examination at 30 days and 60 days post treatment**
20. **Although the follow-up examinations for this specific study only involve 2 occasions, the individuals involved in the study may be available at later intervals to monitor long-term effects and duration of efficacy specifically for the mesenchymal stem cell treatment group for follow-on research studies**

I confirm that I am aware that the account is to be settled in full at the time of discharge from hospital and I acknowledge that I have read these conditions and hold myself bound thereto.

The patient will be part of the following treatment group: (Tacrolimus or Mesenchymal Stem Cells)

___Tacrolimus_____

Patient Number: **VFAH/T/10**

I give my consent in allowing my dog to be a part of the following study and trial:

Date:
Full Name of Owner / Legal Agent:

..... SIGNATURE: .....

Full Name of witness:

..... SIGNATURE: .....

Patient belongings:

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Addendum 3 Permissions

Figure 2.1

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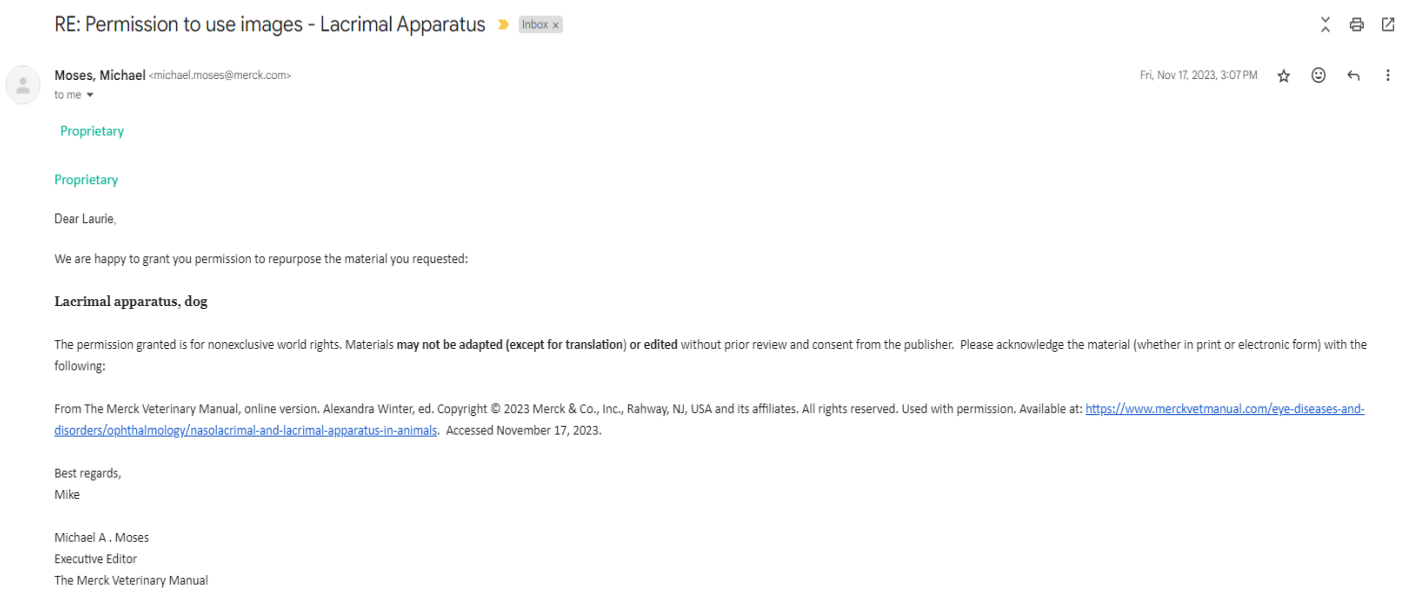
Title:	An eye on the dog as the scientist's best friend for translational research in ophthalmology: Focus on the ocular surface
Type of Use:	Dissertation/Thesis
Order Total:	0.00 USD

View or print complete details of your order and the publisher's terms and conditions.

Sincerely,

Copyright Clearance Center

Figure 2.2



RE: Permission to use images - Lacrimal Apparatus Inbox ×

Moses, Michael <michael.moses@merck.com> Fri, Nov 17, 2023, 3:07 PM
to me

Proprietary

Proprietary

Dear Laurie,

We are happy to grant you permission to repurpose the material you requested:

Lacrimal apparatus, dog

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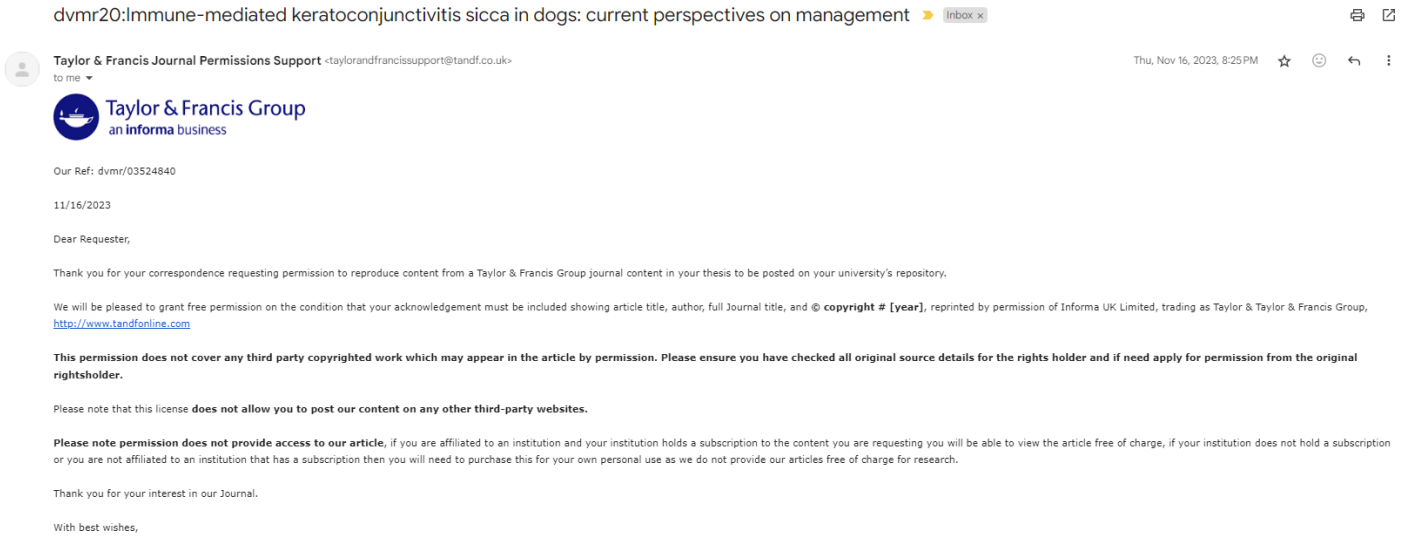
From The Merck Veterinary Manual, online version. Alexandra Winter, ed. Copyright © 2023 Merck & Co., Inc., Rahway, NJ, USA and its affiliates. All rights reserved. Used with permission. Available at: https://www.merckvetmanual.com/eye-diseases-and-disorders/ophthalmology/nasolacrimal-and-lacrimal-apparatus-in-animals. Accessed November 17, 2023.

Best regards,
Mike

Michael A . Moses
Executive Editor
The Merck Veterinary Manual

Figure 2.3



dvmr20:Immune-mediated keratoconjunctivitis sicca in dogs: current perspectives on management Inbox ×

Taylor & Francis Journal Permissions Support <taylorandfrancissupport@tandf.co.uk> Thu, Nov 16, 2023, 8:25 PM
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11/16/2023

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Thank you for your interest in our Journal.

With best wishes,

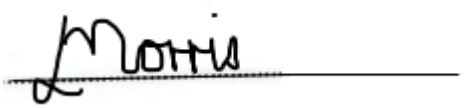
Addendum 4 Data Collection Form

	A	B	C	D	E	F	G	H	I	J
1	Dog	Treatment	Time	STT OD	TBUT OD	IOP OD	STT OS	IOP OS	Notes	
2	1	Tac	0							
3	1	Tac	30							
4	1	Tac	60							
5	2	Tac	0							
6	2	Tac	30							
7	2	Tac	60							
8										
9										
10										
11										

	A	B	C	D	E	F	G
1	Corneal Scoring Sheet						
2	Dog	Treatment	Time	OD	OS	Overall	
3	1	Tac	0				
4	1	Tac	30				
5	1	Tac	60				
6	2	Tac	0				
7	2	Tac	30				
8	2	Tac	60				
9							

Addendum 5 Artificial Intelligence Declaration

I, Laurie Morris , declare that no part of this dissertation was written with the assistance of any artificial intelligence word processing or thesis generating software. The work reflected in this document is my own under the guidance of my supervisors.



Addendum 6 The ARRIVE Guidelines 2.0: Author Checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including:	Materials and methods
		a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated.	
		b. The experimental unit (e.g. a single animal, litter, or cage of animals).	Materials and methods
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.	Materials and methods
		b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	Sample size and study population
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori*. If no criteria were set, state this explicitly.	Experimental Design, Randomisation and Treatments
		b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.	Those with bilateral end-stage KCS were excluded
		c. For each analysis, report the exact value of *n* in each experimental group.	n=9 for Stem Cell patients and n=8 for Tacrolimus patients
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.	Sample size and study population
		b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Sample size and study population
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Materials and methods
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).	STT, TBUT, IOP and corneal scoring
		b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	Schirmer tear test (STT)
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used.	Data collection and data analysis
		b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	Data collection and data analysis
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.	Materials and methods
		b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Materials and methods
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including:	Treatment groups
		a. What was done, how it was done and what was used.	Treatment groups
		b. When and how often.	Treatment groups
		c. Where (including detail of any acclimatisation periods).	Literature review
		d. Why (provide rationale for procedures).	
Results	10	For each experiment conducted, including independent replications, report:	Results
		a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).	Results
		b. If applicable, the effect size with a confidence interval.	

Addendum 7 Faculty of Humanities REC Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Humanities
Fakulteit Geesteswetenskappe
Lefapha la Bomotho

Humanities 100
Since 1919

26 August 2022

Dear Ms LM Morris

Project Title:	The use of allogenic adipose-derived mesenchymal stem cells in the treatment of canine keratoconjunctivitis sicca
Researcher:	Ms LM Morris
Supervisor(s):	Dr LT Odayar
Department:	Companion Animal Clinical Studies
Reference number:	16117574 (HUM011/0722)
Degree:	Masters

Thank you for the application that was submitted for ethical consideration.

The Research Ethics Committee notes that this is a literature-based study and no human subjects are involved. The application has been **approved** on 26 August 2022 with the assumption that the document(s) are in the public domain. Data collection may therefore commence, along these guidelines.

Please note that this approval is based on the assumption that the research will be carried out along the lines laid out in the proposal. However, should the actual research depart significantly from the proposed research, a new research proposal and application for ethical clearance will have to be submitted for approval.

We wish you success with the project.

Sincerely,

Prof Karen Harris
Chair: Research Ethics Committee
Faculty of Humanities
UNIVERSITY OF PRETORIA
e-mail: tracey.andrew@up.ac.za

Addendum 8 Section 20 Letter

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001
Enquiries: Ms. Mama Laing · Tel: 012 319 7442 · Fax: +27 12 319 7470 E-mail: MamaL@dalrrd.gov.za
Reference: 12/11/1/1/2426 (HP) (2705ZY)

Dr Laurie Megan Morris
Veterinary Export Control Office (VECO), Milnerton, Cape Town
22 Lobelia Street
Milnerton
Cape Town
7441
E-mail: morrislaurie7@gmail.com

Dear Dr Laurie Megan Morris,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) – EXTENSION OF THE EXPIRY DATE

Title of research project / study: "The use of allogenic adipose-derived mesenchymal stem cells in the treatment of canine keratoconjunctivitis sicca"

An amendment is hereby granted on the Section 20 approval that was issued for the above mentioned study on 2022-05-12.

1. As requested, the validity of the section 20 approval is extended to 31 October 2025;
2. All other conditions as specified in the Section 20 approval of 2022-05-12 remain in full effect. This includes the validity of laboratory approvals in terms of SANAS and DALRRD.

Kind regards,

DIRECTOR: ANIMAL HEALTH

Date:

2022-10-03

– 1 –

Addendum 9 Declaration of Originality

UNIVERSITEIT VAN PRETORIA
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UNIVERSITY OF PRETORIA

FACULTY OF VETERINARY SCIENCE

DECLARATION OF ORIGINALITY

This document must be signed and submitted with every essay, report, project, assignment, mini-dissertation, dissertation and/or thesis

Full names of student: Laurie Megan Morris

Student number: 16117574

Declaration:

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this dissertation (e.g. essay, report, project, assignment, mini-dissertation, dissertation, thesis, etc.) is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
3. I have not used work previously produced by another student or any other person to hand in as my own.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature of student: .....

Signature of supervisor: .....

Addendum 10 Faculty of Veterinary Science REC Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

01 July 2024

LETTER OF APPROVAL

Ethics Reference No	**REC058-22**
Protocol Title	**The use of allogenic adipose-derived mesenchymal stem cells in the treatment of canine keratoconjunctivitis sicca**
Principal Investigator	**Ms LM Morris**
Supervisors	**Prof GE Zeiler** **Dr LT Odayar**

Dear Ms LM Morris,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC058-22) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. Note: All FVS animal research applications for ethical clearance will be automatically rerouted to the Animal Ethics committee (AEC) once the applications meet the requirements for FVS ethical clearance. As such, all FVS REC applications for ethical clearance related to human health research will be automatically rerouted to the Health Sciences Research Ethics Committee, and all FVS applications involving a questionnaire will be automatically rerouted to the Humanities Research Ethics Committee. Also take note that, should the study involve questionnaires aimed at UP staff or students, permission must also be obtained from the relevant Dean and the UP Survey Committee. Research may not proceed until all approvals are granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

Room 6-6, Arnold Theiler Building
University of Pretoria, Faculty of Veterinary Science
Private Bag X04, Onderstepoort, 0110, South Africa
Tel +27 (0)12 529 8390
Email marie.watson-kriek@up.ac.za
www.up.ac.za

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