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Amniotic membrane transplantation for ocular surface reconstruction in veterinary medicine

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CHAPTER 1

Foreword

1. Foreword

1.1 Amniotic membrane

The fetal membrane is composed by the chorion, allantois and amnion. The AM is the inner layer. It consists of a single layer of ectodermally derived cuboidal to columnar cells, which form the epithelium, a basement membrane that connects with the chorion by a layer of mesenchyme which contains large amounts of collagen (stroma). Amniotic basement membrane and stroma contain cytokines, proteoglycans, collagen type I, III, IV, V and VII, laminin and fibronectin.[7-12] Different interesting properties of the AM have been described. Amniotic basement membrane, by serving as a "transplanted basement membrane", acts as a new healthy substrate that facilitates migration of epithelial cells, reinforces adhesion of basal epithelial cells, promotes epithelial differentiation, and prevents epithelial apoptosis.[8,10] Additionally AM produces various growth factors such as basic fibroblast growth factor, hepatocyte growth factor and transforming growth factor β, that can stimulate epithelialisation and reduce scar formation.[8,10,12] AM also inhibits protease activity,[8,12] has anti-inflammatory,[8,10,12-14] anti-angiogenic[8,10,13] and antifibrotic effects.[8,10,12,15]

Several factors are involved in the anti-inflammatory action: down-regulation through TGFβ-signaling system; preclusion of polymorphonuclear cell infiltration; suppression of pro-inflammatory cytokines, including IL-1α, IL-2, IL-8, interferon λ, tumor necrosis factor-β, TNF-α, basic fibroblast growth factor and platelet derived growth factor.[8,10-14] AM also attracts and sequesters inflammatory cells infiltrating the ocular surface.[8]

Anti-angiogenic effects may be explained by the anti-inflammatory action and by the release of soluble anti-angiogenic factors from the epithelial and mesenchymal cells of amniotic membrane, such as interleukin-1 receptor

antagonist, tissue inhibitors of metalloproteinase (TIMP), collagen 18, IL-10, thrombospondin-1 and pigment epithelium-derived factor (PEDF).[8,10,13]

AM stromal matrix suppresses TGFβ, proliferation and myofibroblastic differentiation of normal corneal, limbal and conjunctival fibroblast.[8,12,15] This action explains why AM helps reduce scars formation after conjunctival and corneal surface reconstruction.[14]

Finally it has been noted that AM has antibacterial and antiviral activities. This is explained by the presence of various compounds that promote anti-microbial immunity, such as interleukins, interferons, TNF-α, activin A, inhibin A, pre-B-cell colony-enhancing factor and leukemia inhibitory factor.[8,10,14]

The non-immunogenicity of the AM was believed to be another important property. It was thought that AM did not express HLA-A, -B, or –DR antigen since after transplantation it did not undergo rejection. Subsequent studies have shown class 1 antigen and co-manifestation of class 1a (HLA-A, -B,-C, -DR) and class 1b (HLA-G and HLA-E) antigen in amniotic epithelium and stroma. The techniques of AM processing and preservation render all the amniotic cells nonviable and hence its immunogenicity is of no consequence.[10,16]

AM may be used either as a graft (inlay) or a patch (overlay) or in multiple layers. In the inlay technique the AM is tailored to the size of the defect and is meant to act as a scaffold for the epithelial cells. The AM is secured with its basement membrane or epithelial side up to allow migration of the surrounding epithelial cells on the membrane.[10,11] In the overlay technique the AM is used akin to a biological contact lens in order to protect the healing surface defect beneath and to act as barrier to protect the cornea from inflammatory cells and proteins in the tear film. The AM is secured with its epithelial side up and it either falls off or is removed.[10,11] In the filling-in technique the entire depth of an ulcer crater is filled with small pieces of AM trimmed to the size of the defect. The orientation of these pieces does not matter. The most superficial piece is placed with the

basement membrane side up and sutured as an inlay graft enabling corneal epithelium to grow over it.[10,11]

In this study we are illustrating the use of amniotic membrane transplantation for ocular surface reconstruction in different diseases in cats, dogs and horses. In the following paragraphs we will briefly describe the ocular conditions that we treated with this procedure.

1.2 Feline corneal diseases

1.2.1 Feline corneal sequestrum

Corneal sequestrum (corneal nigrum, corneal mummification, corneal necrosis or necrotizing keratitis) is a disease unique to the feline species, characterized by a localized necrosis of corneal epithelium and superficial stroma that can progress to the deeper stromal layers.[1-3] The lesion is brown and can show different degrees of vascularization. Brachycephalic cats seem to be predisposed to corneal sequestrum, suggesting that it can occur following chronic exposure of the central corneal.[1,2,4] Other predisposing causes of the lesion are feline herpes virus (FHV-1)[5] and chronic corneal irritation, such as keratitis secondary to palpebral defects (eg. entropion).[2,6] The most effective therapy is still controversial, but most veterinary ophthalmologists treat the lesion with a lamellar keratectomy with or without secondary placement of a pedicle conjunctival graft. The graft seems to reduce the recurrence rate of the sequestrum after keratectomy and can provide a tectonic support in case of deep keratectomy, although it is associated to secondary corneal scars of varying density.[1] Lamellar keratoplasty,[4] corneoconjunctival transposition[2] and penetrating keratoplasty[3] have been described as surgical treatment of feline corneal sequestrum, with a possible improved cosmetic outcome than conjunctival graft.

1.3 Canine corneal diseases

1.3.1 Dermoid

Dermoids are choristoma, or congenital circumscribed overgrowth of microscopically normal tissue in an abnormal place. They are described in different species. The tissue may involve conjunctiva, third eyelid, eyelid margin, limbus or cornea in various combination. In dermoids containing hair follicles, hair grows from the surface, causing conjunctival and corneal irritation, evident as corneal opacity, conjunctival hyperemia and ocular discharge. The mass, depending on its location, can be a source of severe ocular pain. If on the lid margins, it prevents the normal blinking reflex and therefore proper eye lubrication. Dermoids located on the cornea are a physical barrier preventing normal vision. Tratement requires careful surgical excision with conjunctivectomy, combined with keratectomy if they cross the limbus.[34]

1.3.2 Stromal corneal ulcer

A corneal ulcer is any keratopathy in wich there is a loss of epithelium. When this first layer is damaged the surface of the eye is no longer smooth and bacteria can now enter the eye causing the eye to become painful. The corneal ulcer can deepen and widen and involve deeper layers of the cornea.

The cause of a corneal ulcer can be anything that causes an abrasion to the eye. Dogs that rub their face with their paw, scratch to the eye by another animal, thorns, grass seeds, eyelid/cilia abnormalities, tear deficiencies, and foreign objects are all common causes for ulcers.

An ulcer should heal within 7 days and without progression to involve the stroma ("simple ulcer"). Complicated corneal ulcers involved the stroma and/or persist longer than 7 days. Topically medication with antibiotics is indicated for all corneal ulcers.

Ulcers that are rapidly progressive or that have areas of stromal melting, stromal loss, or marked cellular infiltrate are considered complicated and assumed to be

infected. In these cases medical therapy could not be enough, and surgical treatment is suggested. Conjunctival grafts can provide mechanical support for a thin or weakened cornea, supply of serum, which contains anticollagenases and growth factors, source of actively replicating fibroblasts for collagen regeneration in the stroma, delivery of systemic antibiotics. The disadvantage of the conjunctival graft is the evident corneal scar that could induce partial vision impairement.[34] Porcine small intestinal submucosa (SIS) grafts, amniotic membrane grafts, corneaoscleral/coreoconjunctival transposition, lamellar corneal grafts are other surgical options described. [7,34]

1.4 Equine corneal disease

1.4.1 Melting ulcer

Equine corneal ulceration is one of the most important disease of the horse cornea is a potentially sight-threatening disease requiring early clinical diagnosis, laboratory confirmation, and appropriate medical and surgical therapy.[33]

Infection by bacteria and fungi should be considered in every corneal ulcer. Bacteria living on the surface of the cornea are able to attach to the corneal cells once the epithelium has been damaged. Many bacterial species cause corneal ulcers in horses, but Gram-negative bacteria such as *Pseudomonas* and Gram-positive bacteria such as *Staphylococcus* and *Streptococcus* are particularly feared. Infection of corneal ulcers in horses is common. Bacteria recruit white blood cells that produce enzymes to the ulcer site; these enzymes digest collagen in the stroma and greatly speed the progression of an ulcer. Enzymatic activity in corneal ulcers is referred to as "melting" and gives a grayish, liquefied appearance around the ulcer. Melting is a serious problem for the horse cornea.[31,33]

Corneal ulcers are painful and stimulation of corneal sensory nerves causes painful inflammation of the iris (anterior uveitis). Other common clinical signs of corneal ulcers include tearing and corneal cloudiness.

Corneal ulcers in horses must be aggressively treated in order to preserve vision. Subpalpebral tubing treatment systems might be necessary for medicating horses with painful eyes. The primary treatment for bacterial corneal ulcers is intensive administration of antibiotics. Broad-spectrum antibiotics are usually administered three to eight times per day to kill bacteria in the eyes of horses with ulcers. Prevention of collagen breakdown and ulcer progression is also important in ulcer therapy. Enzymes derived from white blood cells in the tears can be powerful forces in the destruction of the corneal stroma, and can cause rapid deepening of ulcers. Topical corticosteroids increase this damaging enzyme activity and should not be used in the treatment of corneal ulcers.[33]

Serum from the blood of the horse contains proteins with anti-melting activity and can be placed in the eye hourly for melting ulcers in horses. Ethylenediaminetetraacetic acic (EDTA 0.2%) and Acetylcysteine (5-10%) can also be used topically for its melting enzyme-inhibiting properties. Treatment of the anterior uveitis found with corneal ulcers in horses is critical. Topical 1% atropine is usually given to relieve pain and dilate the pupil. Non steroidal anti-inflammatory drugs are used orally, intramuscularly, or intravenously to relieve eye pain and inflammation. To increase lost corneal thickness and strength, deep corneal ulcers threatening rupture of the eye and corneas that have ruptured require conjunctival flap placement or corneal transplantation surgery.[33] Porcine small intestinal submucosa (SIS) grafts, amniotic membrane grafts, corneaoscleral/coreoconjunctival transposition, lamellar corneal grafts are other surgical options described. [8,31,33]

1.4.2 Fungal ulcer

Keratomycosis refers to "kerato," meaning corneal disease, and "mycosis" referring to fungal infection. The term keratomycosis refers to any corneal disease caused by fungus; these diseases include ulcers, abscesses, and iris

prolapses. Several species of septate filamentous fungi are common to the equine eye (*Fusarium, Aspergillus,* and *Penicillium*).[33]

Fungal keratitis are more common and the clinical signs are the most severe in horses living in warm, humid geographic regions Seasonality of infection in Italy shows that fungal ulcers occur with the majority of cases in May through November.

The pathogenesis of fungal ulcers (ulcerative keratomycosis) commonly begins with different severity of corneal trauma resulting in exposure of the stroma, and stromal invasion by the fungal organisms living on the surface of the horse eye or in the environnement. Further corneal destruction results from the release of enzymes from the fungi, white blood cells, and corneal cells.[33]

Ulcers infected with fungi range from minor corneal epithelial abrasions to superficial plaques, to extremely deep, severe ulcers. Diagnosis of keratomycosis is based on finding fungal hyphae or yeast on cytologic (cellular) examination of a corneal scraping, culture of the corneal lesion, or surgical biopsy. Fungal hyphae are frequently found deep in the equine cornea rather than on the surface.[33]

Treatment must be directed against the fungi as well as against the corneal and intraocular inflammatory responses that occur following fungal replication and death of the hyphae. Anti-fungal drug treatment is often required for an extended period to achieve complete fungal destruction and resolution of the clinical . Antibiotic treatment is also indicated with keratomycosis as concurrent bacterial infection is relatively common. Topical corticosteroids are contraindicated in keratomycosis in horses as they can worsen the disease by enhancing fungal replication, predisposing the cornea to further fungal infection, and decreasing the effectiveness of anti-fungal drugs.[33] Combined medical and surgical therapy is indicated if ulcers are extremely deep, if they are not responding to medical treatment, or if they worsen in spite of medical treatment.

Surgeries for keratomycosis include conjunctival grafts, amniotic membrane transplantation and full-thickness corneal transplantation.[8,33]

1.4.3 Immune-mediate keratitis

Immune-mediate keratitis (IMMK) is characterized by chronic nonulcerative keratitis with cellular infiltrate and corneal vascularization. Other characteristic features of this disease are lack of secondary uveitis or severe discomfort, no micro-organisms and therapeutic response to anti-inflammatory medications.[33,35] The possible pathogenesis of IMMK in horses is that the immune system has recognized a self-antigen in the cornea (i.e. molecular mimicry) or a foreign protein or organism antigen within the cornea. An underlying infectious agent may be either the inciting or perpetuating cause (or both) in many horses with IMMK. This organism may be directly inciting active inflammation or may have induced immunologic cross-reaction with self-antigen in the cornea.[35] Both medical and surgical therapies could be indicated. Topical steroids or cyclosporine could be effective.[33] Removal of this inciting antigen via keratectomy may eliminate the source of the immune reaction and stop the inflammatory process.[35]

1.4.4 Corneal edema

Normal corneal hydration reprents the balance between the fluid leak across the corneal endothelium and the extrusion of fluid via the endothelial metabolic pump. Corneal edema is characterized by a bluish opacity that could cause visual impairment and potentially recurrent corneal ulcers resulting from rupture of epithelial bullae. The possible causes of corneal edema could be: primary endothelial degeneration, glaucoma or uveitis. Topical hyperosmotic agents are descrive for therapy, but they don't induce a resolution of the clinical signs.[33]

1.4.5 Corneal Squamous Cell Carcinoma

Squamous cell carcinoma (SCC) is the most common tumor affecting the eye in horses. The way the tumor grows and spreads might be related to exposure to the ultraviolet (UV) component of solar radiation, periocular pigmentation, and an increased genetic susceptibility to tumor formation. The UV component is the most plausible carcinogenic agent associated with SCC, as it targets the tumor suppressor gene p53 that is altered in equine SCC.[30,33]

Horse breeds with the least ocular and periocular pigmentation appear to be at increased risk for ocular SCC. Draft horses have a higher prevalence, followed by Appaloosas and Paints, with the least prevalence found in Arabians, Thoroughbreds, and Quarter Horses.[30,33] The prevalence of SCC increases with age.

The cornea, conjunctiva, and limbus are the most commonly affected area. The appearance of corneal SCC varies, depending on the duration of the lesion.[7,30,33] These tumors are generally pink, fleshy masses that are raised or flat. Diagnosis is by biopsy, and treatment selection depends on tumor location, tumor size and extent of tumor invasion. Surgical excision alone could not be enough and should be followed by either radiation, cryotherapy, hyperthermia, or intralesional chemotherapy to prevent recurrence.[7,8,30,33]

CHAPTER 2

Objectives

2. Objectives

The use of amniotic membrane (AM) for corneal surface reconstruction is usually associated to a high degree of corneal clarity both in human and animal.[7,8] The purpose of this study is to evaluate clarity of the cornea (esthetic outcome) and the quality of the vision (functional outcome) after equine amniotic membrane transplantation for ocular surface reconstruction in cats, dogs and horses affected by different ocular diseases. In cats the work is a pilot study for the use of this surgical technique after lamellar keratectomy for corneal sequestra.

CHAPTER 3

Materials and Methods

3. Materials and methods

3.1 Amniotic membrane preparation

The AM was obtained from the placenta of a mare undergoing cesarean section. The AM was dissected from the chorion, cleaned with saline solution first, then with povidone iodine solution 0.1% and finally with a gentamicin solution 0.2%. All 3 solutions were alternated 3 times. The membranes were placed on a nitrocellulose paper with the epithelial surface up, cut into 5 x 5 cm pieces and stored in 98% glycerin at room temperature. A sample of the membrane was placed in formalin and submitted for histopathologic examination while a second sample was submitted for bacterial culture to confirm asepsis. The median time of storage of the AM before use was 66.15 days (range 1-184 days).

The histopathologic appearance of the AM at different times of preservation (fresh, after 8 weeks and after 16 weeks) was also compared.

3.2 Feline cases

AM transplantation was performed in six cats (seven eyes) diagnosed with corneal sequestra. Data collected for each case included breed, gender, age, eye affected, clinical signs and information about the sequestrum (size, depth, location and vascularization). Presence of ophthalmic lesions potentially related to corneal sequestrum formation such as corneal erosion or entropion were also recorded. Complete blood count (CBC), serum chemistry, urinalysis, feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) tests (ELISA) were performed in all cases.

The affected eye was routinely prepared for ocular surgery with 0.1% povidone iodine solution. A lamellar keratectomy was performed in all cats. A curvilinear incision was made using a #64 Beaver blade, following along and outside the margin of the corneal lesion. A Martinez corneal dissector (Storz, St Louis, MO, USA) was used to separate the corneal lamellae and create a corneal flap,

containing the entire lesion. Westcott tenotomy scissors were then used to sharply dissect the flap from the remainder of the cornea. The corneal defect was covered by an equine AM graft, that was cut to size, placed over the entire keratectomy bed and then sutured with AM stroma against the exposed corneal stroma to the margin of the keratectomy with 8-0 Vicryl (Vicryl®, Ethicon) in an interrupted or continuous pattern. The keratectomy sample and a small piece of the AM used for each eye were placed in formalin and submitted for histopathologic examination. Tobramycin (Tobral®, Alcon Italia) q 8 hrs was given topically after surgery until healing occurred and oral doxycycline (Ronaxan®,Merial Italia) 10mg/Kg q 24 hrs was administered for 10 days.

3.3 Canine cases

AM transplantation was performed in three dogs (three eyes) diagnosed with stromal corneal ulcer or dermoid. Data collected for each case included breed, gender, age, eye affected, clinical signs and information about the lesion (size, depth, location and vascularization). Complete blood count (CBC), serum chemistry and urinalysis were performed in all cases.

The affected eye was routinely prepared for ocular surgery with 0.1% povidone iodine solution. The excision of the dermoids was performed in a similar manner as described for feline cases but extending on the conjunctiva and on the lateral cantus when affected. In the corneal stromal ulcer the necrotic and infiltrated tissue around the lesion was removed by #64 Beaver blade and Westcott tenotomy scissors. In all cases the corneal/conjunctival defect was covered by an equine AM graft, that was cut to size, placed over the entire keratectomy bed and then sutured, with AM stroma side down, to the margin of the keratectomy/conjunctivectomy with 8-0 Vicryl (Vicryl®, Ethicon) in an interrupted or continuous pattern. The cantus was sutured with a 6-0 Vicryl (Vicryl®, Ethicon) in a simple interrupted manner with a "eighth shape" knot on the margin. The removed lesions and a small piece of the AM used for each eye

were placed in formalin and submitted for histopathologic examination. Tobramycin (Tobral®, Alcon Italia) q 8 hrs was given topically after surgery until healing occurred and oral doxycycline (Ronaxan®,Merial Italia) 10mg/Kg q 24 hrs was administered for 10 days.

3.4 Equine cases

AM transplantation was performed in eleven horses (eleven eyes) diagnosed with corneal melting ulcer, corneal fungal ulcer, corneal edema, immune-mediated keratitis or corneal squamous cell carcinoma.. Data collected for each case included breed, gender, age, eye affected, clinical signs and information about the lesion (size, depth, location and vascularization). Complete blood count (CBC), serum chemistry and urinalysis were performed in all cases.

The affected eye was routinely prepared for ocular surgery with 0.1% povidone iodine solution. A lamellar keratectomy was performed in all cats extending to the limbus and conjunctiva in the SCC cases. A curvilinear incision was made using a #64 Beaver blade, following along and outside the margin of the corneal lesion. A Martinez corneal dissector (Storz, St Louis, MO, USA) was used to separate the corneal lamellae and create a corneal flap, containing the entire lesion. Westcott tenotomy scissors were then used to sharply dissect the flap from the remainder of the cornea. The corneal defect was covered by an equine AM graft, that was cut to size, placed over the entire keratectomy bed and then sutured with AM stroma against the exposed corneal stroma to the margin of the keratectomy with 8-0 Vicryl (Vicryl®, Ethicon) in an interrupted or continuous pattern. The keratectomy sample and a small piece of the AM used for each eye were placed in formalin and submitted for histopathologic examination. Flunixin meglumine (1mg/Kg/24 hrs for 3 days, then 0,5 mg/Kg/24 hrs for 5-7 days) was administered intramuscularly. Tobramycin q 8 hrs, atropine 1% q 12 hrs and itraconazole 1% q 6-8 hrs (for fungal ulcers only) were given topically after surgery until healing occurred.

CHAPTER 4

Results

4. Results

4.1 Amniotic membrane

Histopathologic examination of the AM showed that the epithelium survives for up to 60 days after preservation. Fresh AM (1 week of preservation in glycerol) was composed of a single layer of cuboidal epithelial cells resting on a thin basement lamina that was, in turn, connected to the amniotic mesoderm. The latter had an acellular compact layer, closest to the epithelium, and a deeper layer in which a network of loosely dispersed fibroblast-like cells was recognizable (Fig 1a). After 60 days of preservation in glycerol the epithelial cell lining was still recognizable although it appeared discontinuous with shrinkage of single epithelial cells, while mesoderm was generally more dense (Fig. 1b). After 4 months (120 days) of preservation the amniotic epithelium was no longer recognizable and the stroma appeared more compacted and dehydrated (Fig. 1c).

No correlation was found between the state of preservation of AM and the outcome of surgical procedure.

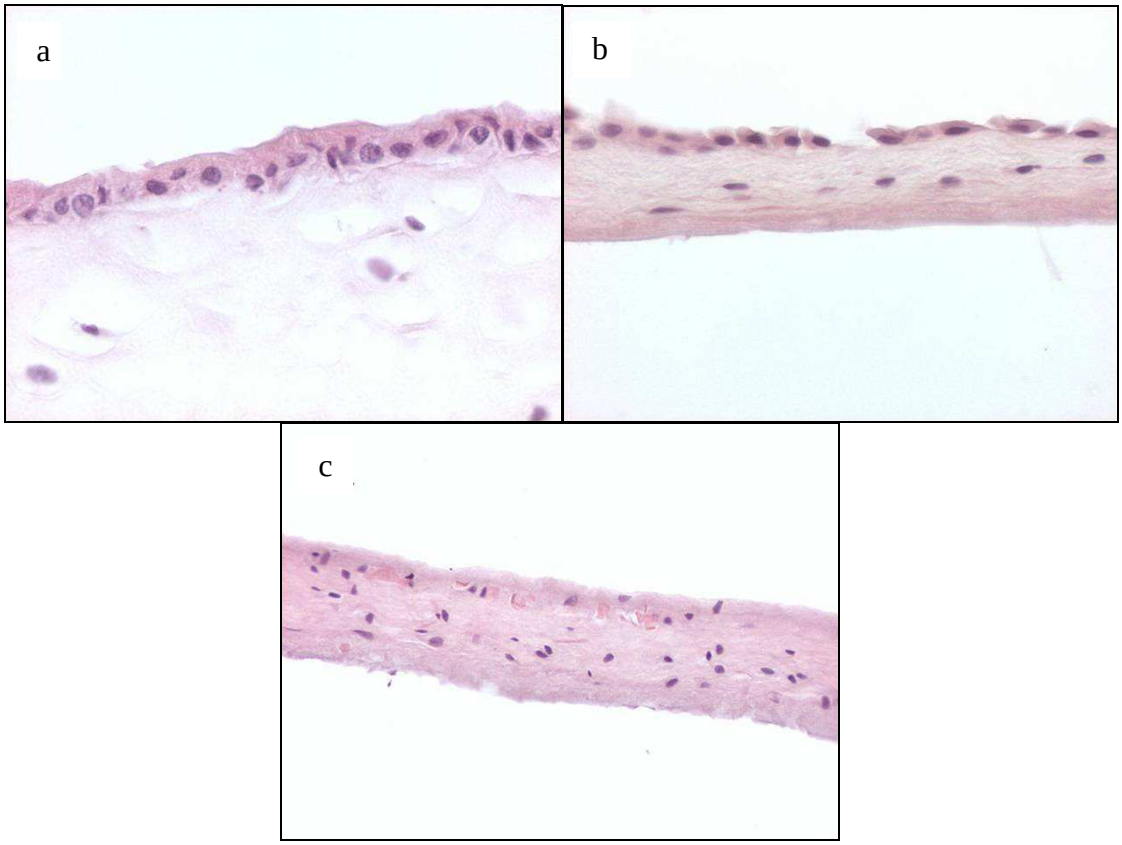

Figure 1. Histological sections (6 mm) of amniotic membrane at different times of preservations (a) fresh amniotic membrane, with an intact epithelium layer. (b) amniotic membrane after 8 weeks of preservation in glycerol. The epithelial layer is discontinuous and the stroma is compact discontinuous. (c) amniotic membrane after 16 weeks of preservation. The epithelium is totally disappeared the stroma is dehydrated and compact. H&E, original magnification 400x. *(Courtesy Dr. Chiara Giudice)*

4.2 Cats

The sequestrum affected the right cornea in one cat, the left cornea in four cats and both corneas in one cat. Breeds affected included four Persian and two domestic shorthair cats. There were four spayed females, one intact male and one castrated male. The median age at the time of treatment was 5.3 years (range 2-12 years). Additional findings included medial entropion of the lower eyelid in four cats and persistent epithelial erosion around the sequestrum in one eye. The depth of one sequestrum was less than 1/3 of the corneal thickness, three were

between 1/3 and 2/3 and three were more than 2/3 corneal thickness depth. Corneal vascularization was absent in two eyes, moderate in two eyes and severe in three eyes (Fig. 2a). One cat was FeLV positive and one was leukopenic because of myelosuppression of unknown origin (Table 1).

After surgery no blepharospasm was noted. In all the eyes there was an epithelial proliferation over the AM within the first 2-4 days after surgery (Fig 2b). In five eyes there was a second phase of corneal granulation tissue proliferation over the next 2-5 weeks (Fig. 2c) and a third phase of corneal clearing in the following weeks. During this last phase an almost complete regression of the preexisting vascularization and granulation tissue was noted with excellent corneal transparency (Fig. 2d). One eye showed the formation of a brown area in the center of the AM (Fig. 3 a-b-c) in the first weeks after transplantation. The corneal sequestrum of this eye was less than 2/3 of the corneal thickness deep and it was completely excised. The lesion involved approximately 80% of corneal surface and the proliferation of the granulation tissue took some time to invade all the AM. The brown area was suspected to be partial necrosis of the last vascularized point that gradually disappeared with the regression of the granulation tissue (Fig. 3d).

Two eyes had complications. In one cornea (case 2, table 1) partial necrosis and rejection of the AM developed two weeks after surgery. This patient was leukopenic and the histopathologic examination of the sequestrum of this eye revealed large aggregates of bacterial cocci and intense neutrophilic infiltration (Fig 4b). This was in contrast to the more typical corneal sequestra composed of large areas of acellular, tan collagen fibers only occasionally demarcated peripherally by leukocytes (Fig 4a). The cornea was epithelialized after rejection of the AM and the patient was lost to follow up for 3 months. After this period the cat was re-presented with a descemetocele in the same area of the previous AM necrosis; a conjunctival pedicle graft was performed at this time.

In a second eye (case 5, table 1) a corneal perforation occurred under the AM two weeks after surgery. The sequestrum of this cornea was deep and without vascularization. A conjunctival pedicle graft was performed to repair the lesion.

Table 1. Signalment, clinical parameters and surgical outcomes in 6 cats (7 eyes) treated with lamellar keratectomy and amniotic membrane transplantation

N°	breed	Age (yrs)	gender	eye	depth	vascularization	Eye disease	histopath	Clinical outcome
1	Persian	2	fs	os	p	++	entropion	typical	good
2	Domestic shorthair	12	fs	od	p	++		Bacteria ++	necrosis and rejection
3	Persian	6.5	m	os	m	+	entropion	typical	good
4	Domestic shorthair	2	fs	os	m	+	ree	typical	good
5	Persian	6.5	fs	od	p	-	entropion	typical	corneal perforation
				os	s	-	entropion	typical	good
6	Persian	3	mc	os	m	++		typical	good

Depth: s: < 1/3 corneal stroma; m: >1/3, < 2/3; p≥ 2/3

fs =female spayed

m= intact male

mc= male castrated

os= left eye

od=right eye

++ = severe

+ = moderate

- = absent

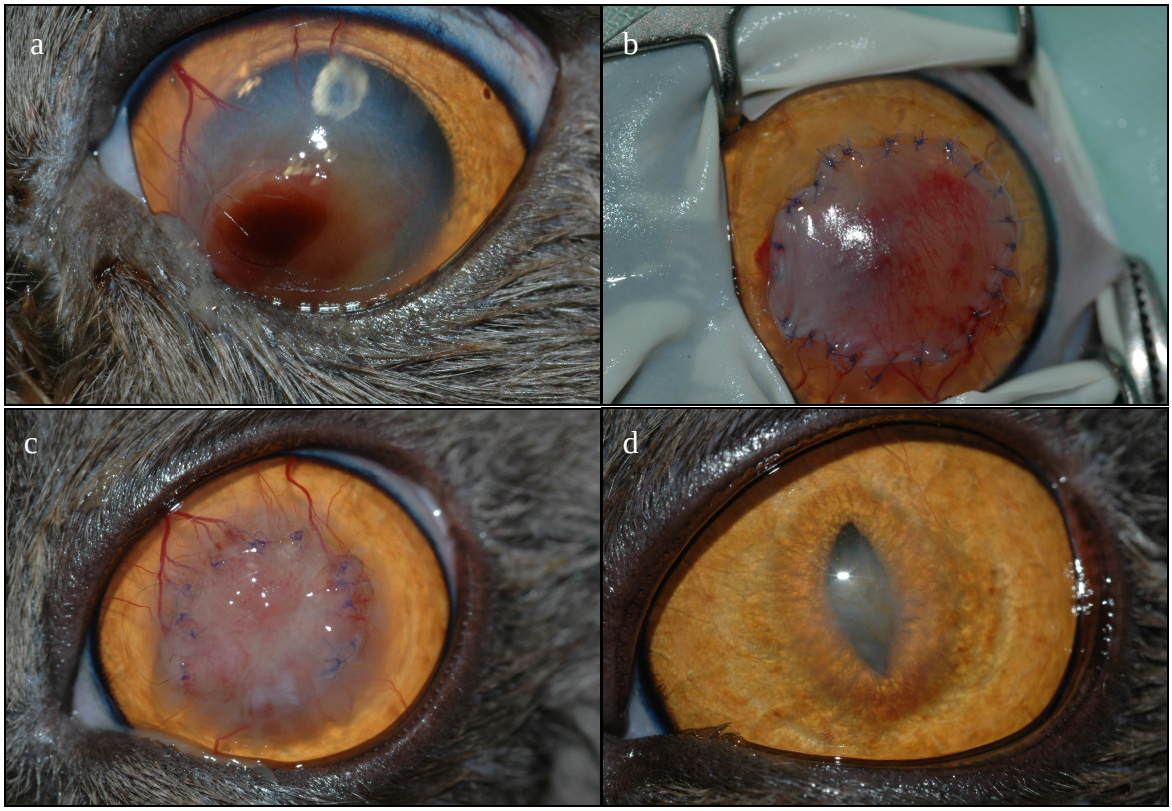

Figure 2. (a) Preoperative appearance of the corneal sequestrum in case 1. (b) Immediate postoperative appearance of amniotic membrane transplant. A peripheral corneal vascular response and vascularization of the deep stroma in keratectomy site may be seen. (c) Appearance of the eye 3 weeks postoperatively. A central corneal granulation tissue developed on the AM. (d) Appearance of the eye 12 weeks postoperatively. Substantial clearing of the graft and resolution of the pre-existing corneal vascularization is noted.

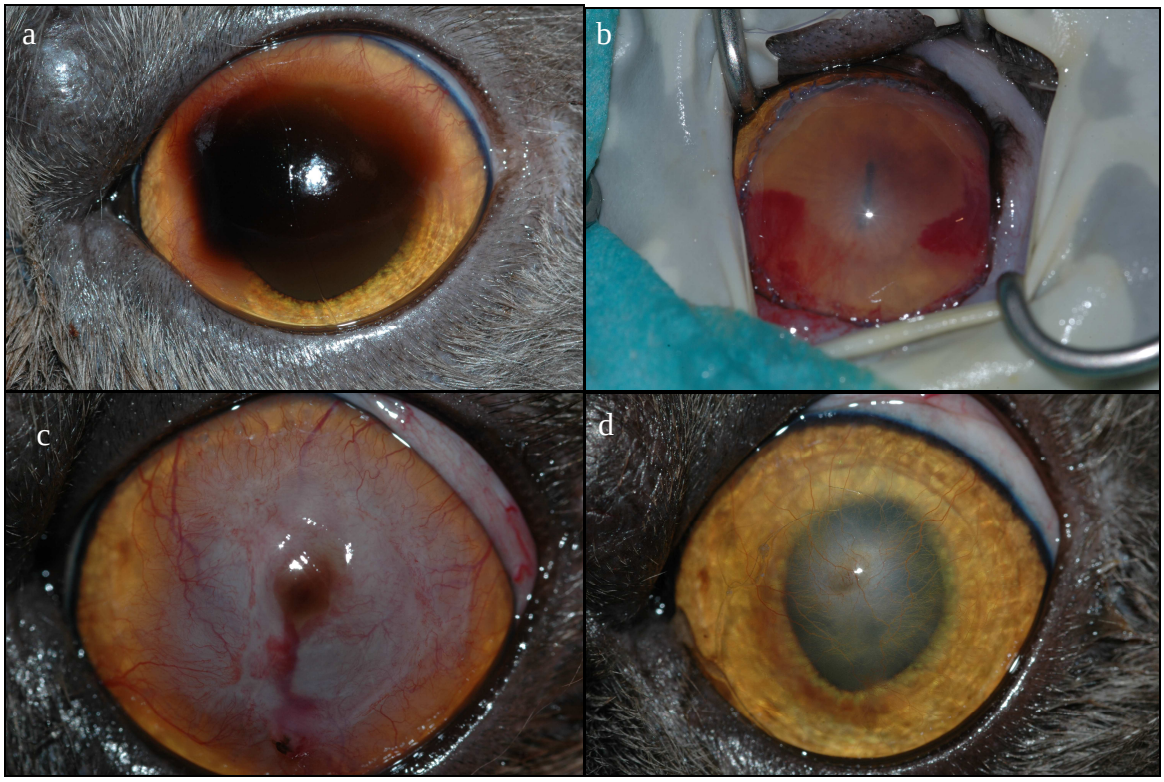

Figure 3. (a) Preoperative appearance of the corneal sequestrum in case 3. (b) Immediate postoperative appearance of the amniotic membrane transplant. (c) Appearance of the eye 5 weeks postoperatively. A granulation tissue developed on the AM with a peculiar brown area in the center; this area could be a partial necrosis in the last point that was vascularized. (d) Appearance of the eye 20 weeks postoperatively. Progressive regression of the granulation tissue and the necrotic portion of the AM.

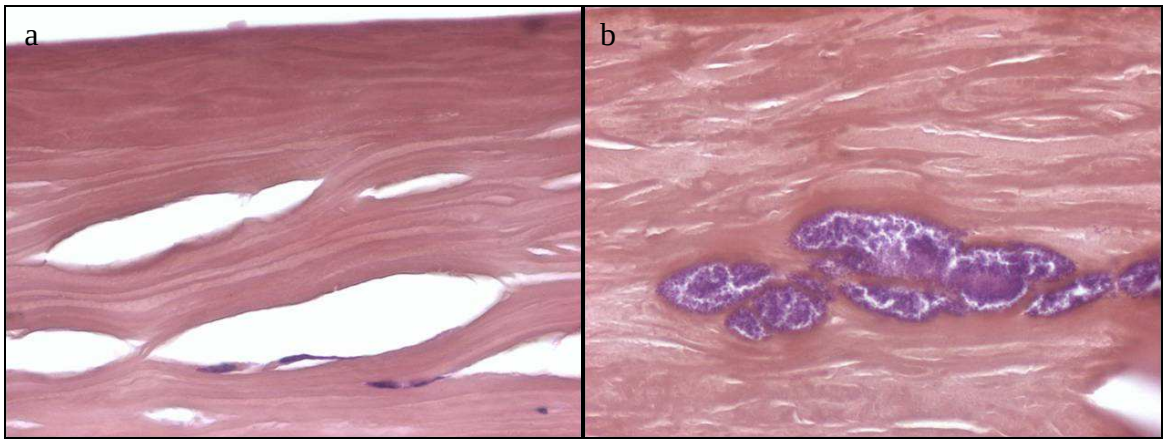

Figure 4. Histological sections (6 mm) (a) typical corneal sequestrum: degenerated corneal fibrils with a characteristic brown pigmentation and sparse spindle cells; (b) corneal sequestrum of case 3: large aggregates of coccoid bacteria are present among fibrils. H&E, original magnification 400x. *(Courtesy Dr. Chiara Giudice)*

4.3 Dogs

One dog was a 5 year old, female Shitzu, affected by stromal corneal ulcer in the right cornea. The ulcer was infected, with moderate vascularization and of more than 2/3 of corneal thickness deep.

The other two dogs were affected by lateral corneo-conjunctival dermoid in the right eye. They were two German shepherds, one male and one female of 6 and 10 months of age respectively. In both dogs the dermoids extended to the lateral cantus.

Immediately after surgery no discomfort was noted. In all the eyes there was an epithelial proliferation over the amnion within the first 2-4 days after surgery, followed by a second phase of corneal granulation tissue proliferation in the next 2-5 weeks and a third phase of corneal clearing in the following weeks. The granulation tissue was particularly evident on the conjunctival area of the excised dermoid (Fig 5). During the last phase an almost complete regression of the preexisting vascularization and granulation tissue was noted, with a good corneal transparency in all the patients.

Figure 5. (a) Preoperative appearance of the corneo-conjunctival dermoid in a dog. (b) Immediate postoperative appearance of amniotic membrane transplant and lateral cantoplasty after excision of the dermoid. (c) Appearance of the eye 1 week postoperatively. A lateral corneo-conjunctival granulation tissue developed on the AM. (d) Appearance of the eye 3 weeks postoperatively. A clearing of the graft and no recurrence of dermoid are noted.

4.4 Horses

Five of the eleven horses were castrated male, four female and two foals, one male and one female. Breeds affected included: 1 paint, 1 appaloosa, 1 IRL, 1 KWPN, 1 French trotters, 2 Dutch, 2 Italian trotters and 2 thoroughbreds. Age ranged from 4 months to 19 years (median 7.5 years). Three patients were treated for melting ulcer (Fig 6), four for fungal ulcer (Fig 7), two for corneo-conjunctival squamous cell carcinoma (SCC) (Fig 8), one for immune-mediated keratitis (IMMK) and one for chronic corneal edema. In the eyes with fungal ulcer amniotic membrane was rejected after a median of 9 days (range 7 to 15 days), leaving a stromal corneal defect, not infected and partially epithelized in three patients. In the two foals, treated for melting ulcer, the amnion was rejected rispectively at 2 and 3 weeks from surgery, leaving an epithelized corneal scar. In the others patients the AM was epithelized in the first week after surgery and progressively vascularized and integrated in the cornea. Corneal scar was mild in seven eyes, moderate in two and severe in two. In these last two subjects we had some complications: one eye, seriously affected by deep fungal ulcer, had an endothelitis with consequent secondary uveitis and phthisis bulbi; in the seconde eye a corneal perforation developed under the amnion. In this case we performed a conjunctival pedicle graft. Visual function was good in seven eyes, subtle in two, bare in the eye with corneal edema, for partial persistence of the edema, and absent in the phthisic eye.

Table 2. Signalment, clinical parameters and surgical outcomes in 11 horses treated with lamellar keratectomy and amniotic membrane transplantation

N°	breed	Age (yrs)	gender	eye	diagnosis	Corneal scar	complication	vision	FU (months)
1	paint	8	mc	os	SCC	mi		+++	10
2	IRL	9	mc	od	edema	mi		+	5
3	Italian trotter	13	mc	os	Fungal ul.	s	Endothelitis Phthisis b.	-	3
4	Italian trotter	3	f	os	melting	mi		+++	12
5	Frech trotter	10	f	od	Fungal ul.	mi		+++	4
6	Dutch	10	mc	os	Fungal ul.	s	perforation	++	5
7	Dutch	8	f	os	Fungal ul	mi		+++	10
8	appaloosa	19	f	os	SCC	mo		++	8
9	KWPN	9	mc	os	IMMK	mi		++	3
10	thoroughbreds	0,33	m	od	melting	mo		+++	1
11	thoroughbreds	0,33	f	od	melting	mo		+++	1

m= intact male

mc= male castrated

os= left eye

od=right eye

mi= mild

mo = moderate

s = severe

+++= good

++= subtle

+= bare

-= absent

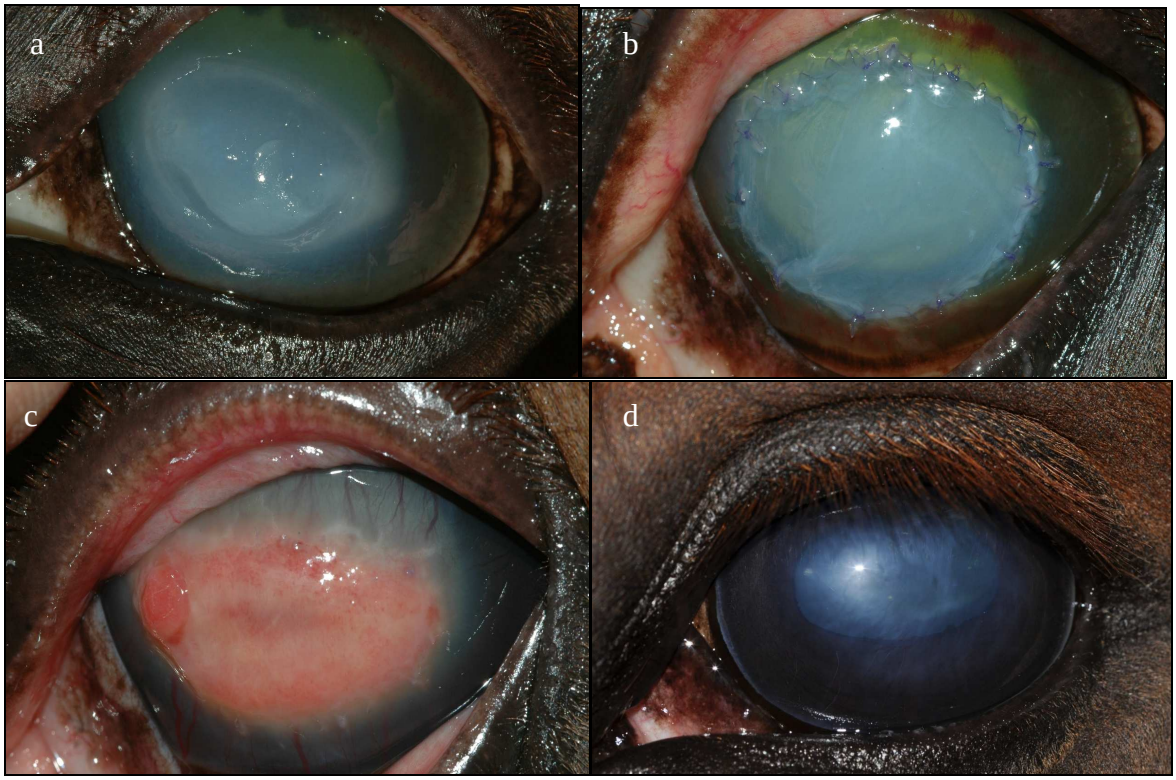

Figure 6. (a) Preoperative appearance of the corneal meltimng ulcer in case 4. (b) Appearance of the amniotic membrane transplant 2 days after surgery. (c) Appearance of the eye 1 month postoperatively. A central corneal granulation tissue and neovascularization developed on the AM. (d) Appearance of the eye 12 months postoperatively. Substantial clearing of the graft and resolution of the pre-existing corneal vascularization is noted. *(Courtesy Dr. Riccardo Stoppini)*

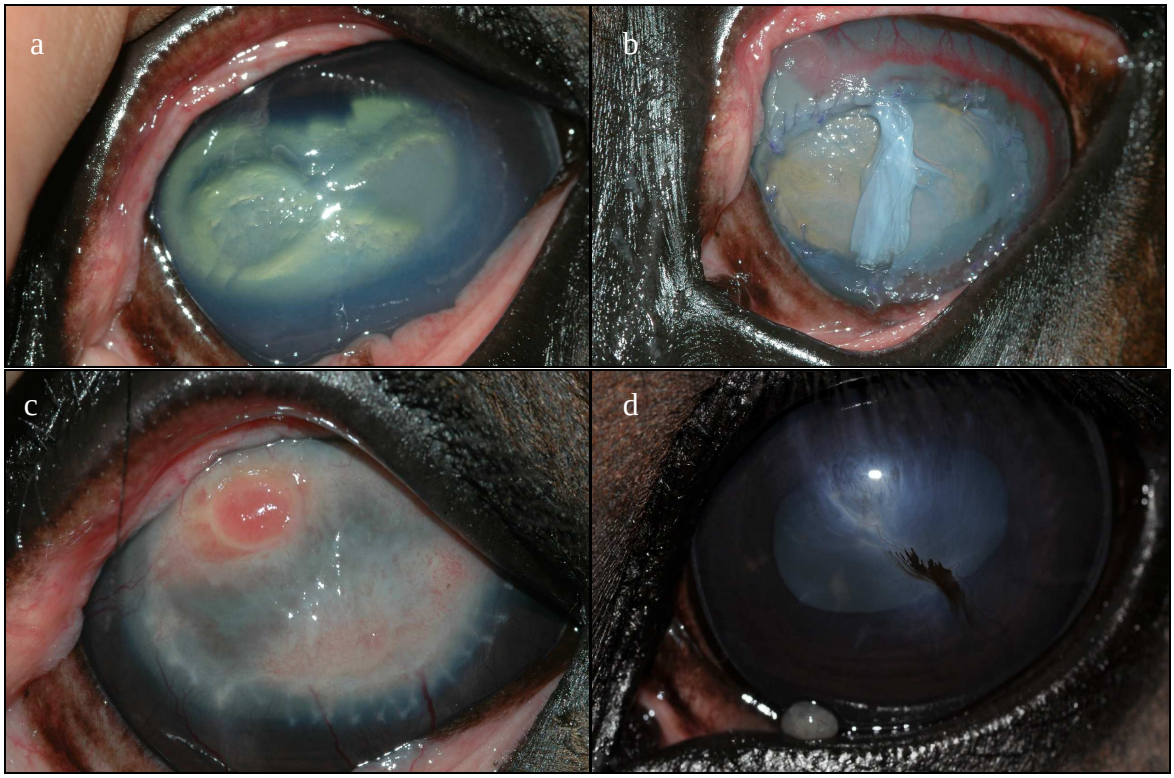

Figure 7. (a) Preoperative appearance of the fungal ulcer in case 7. (b) Appearance of amniotic membrane transplant rejection 10 days after surgery. A peripheral corneal vascular response may be seen. (c) Appearance of the eye 1 month postoperatively. A central corneal granulation tissue developed. (d) Appearance of the eye 10 months postoperatively. Substantial clearing of the cornea and resolution of the pre-existing corneal vascularization is noted. *(Courtesy Dr. Riccardo Stoppini)*

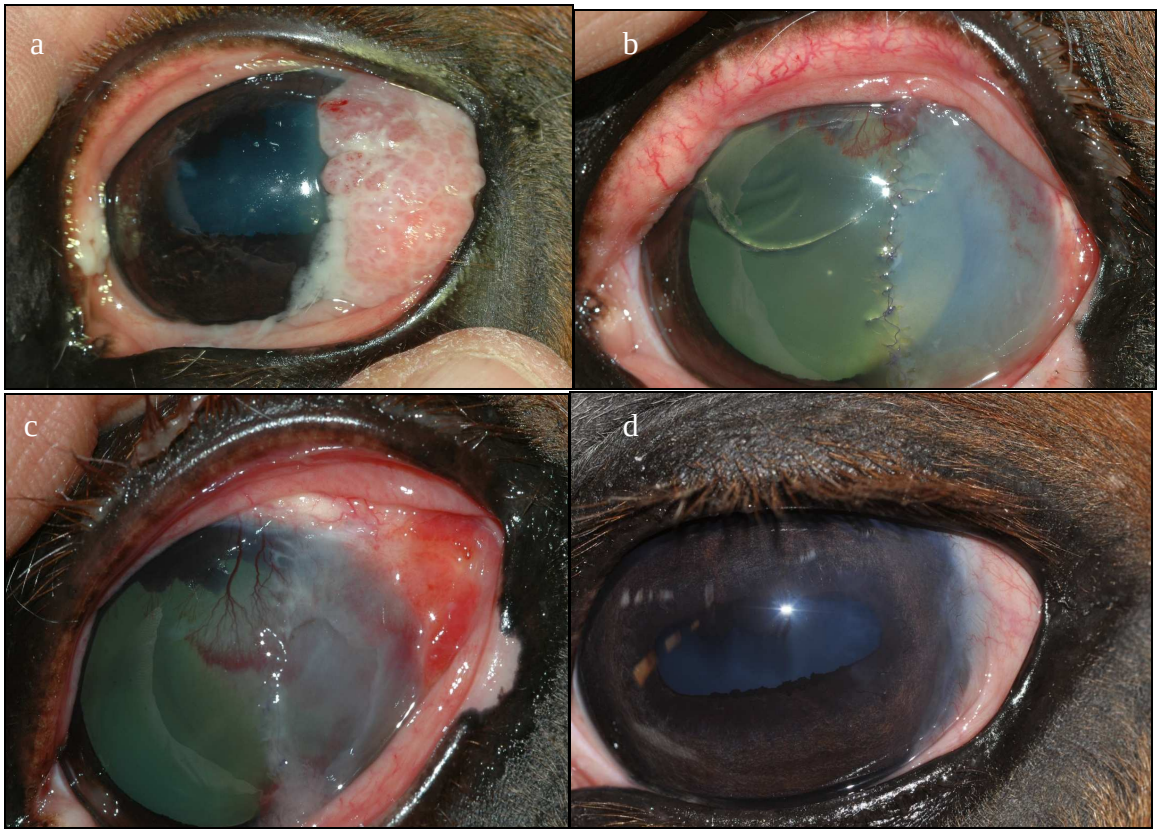

Figure 8. (a) Preoperative appearance of the corneo-conjunctival squamous cell carcinoma in case 1. (b) Appearance of amniotic membrane transplant 4 days after surgery (c) Appearance of the eye 3 weeks postoperatively. Granulation tissue developed on the AM, particularly on the conjunctival area. (d) Appearance of the eye 10 months postoperatively. Substantial clearing of the graft is noted, there is no sign of recurrence. *(Courtesy Dr. Riccardo Stoppini)*

CHAPTER 5

Discussion and conclusion

5. Discussion and conclusion

AM transplantation (AMT) has been widely described in human ophthalmology for the reconstruction of corneal and/or conjunctival surface in different diseases, such as: ocular surface neoplasia,[11] pterygium,[11,17,18] chemical and thermal burns,[11,19,20] cicatrizing conjunctivitis,[11] symblepharon release,[11,17] bleb leakage,[11] filtering surgery,[11,21] persistent epithelial defects,[11,22] non healing stromal ulcers,[11] deep stromal ulcers and descemetoceles,[23] neurotrophic keratopathy,[24] limbal stem cells deficiency,[25] bullous keratopathy,[11,26] infectious keratitis,[27] Stevens Johnson syndrome,[28,29] melting ulcers,[11] scleral melt[11] and band keratopathy.[11] In veterinary ophthalmology AMT has been described for the treatment of ocular surface neoplasia,[7,8,30] bullous keratopaty,[8] melting ulcers,[7,8,31] symblepharon,[7] immun-mediated keratitis[8] and in conjunction with penetrating keratoplasty.[8]

To the authors knowledge there is no report in the literature about the use of AMT as a treatment for feline corneal sequestrum. When compared with other surgical techniques proposed in the literature for corneal sequestrum, AMT can be considered among those with optimal results. Specifically, lamellar keratectomy with secondary placement of a pedicle conjunctival graft has been used by some authors[1] and seems to facilitates healing by providing a direct blood supply to the lesion and by providing tectonic support. It also seems to reduce the recurrence rate of the sequestrum after keratectomy, however it has been associated with secondary corneal scarring of varying density.[1] Lamellar keratoplasty has been described by Pena et al. in 6 eyes that had an optimal cosmetic outcome and no evidence of recurrence of the sequestra for a 4-30 months follow-up periods.[4] Corneoconjunctival transposition has been reported in17 cases by Andrew et al. with minimal scarring from the transposed limbus and no recurrence for a 30 days – 7 years follow-up time. [2] Heterologous

penetrating keratoplasty has also been described in one case for a dense, full-thickness corneal sequestrum.[3] However, in the case reported this technique had a poor outcome. At 9 days postoperatively, the cornea had severe edema, most likely due to diffuse endothelial graft rejection, although at 16 months postoperatively, the cornea had a faint central nebula at the site of surgery. [3] In our case series a successful outcome was obtained with AMT in five out of seven eyes: the AM epithelialized quickly and, after the fibrovascular invasion of the AM, all the corneas had good transparency, with no sequestrum recurrence detected in our follow-up periods.

In two cases we had complications. In one eye there was a partial necrosis of the amnion. This patient was leucopenic and the sequestrum showed a very high bacterial invasion and neutrophilic infiltration. This could justify a higher proteinase activity that couldn't be controlled by the AM. The same bacterial contamination could have caused a melting process in the cornea and the formation of the descemetocele after three months from the AM necrosis.

Another case showed a perforation under the AM. In 2008 M. Kaup *et al.* described a case series of AMT where descemetocele and spontaneous corneal perforation occurred in 25% of the patients. To the authors these complications could be explained by the inhibiting influence of AM on corneal fibroblasts. The suppression of the myofibroblast differentiation by AM might explain the risk of corneal thinning and perforation after AMT in patients with corneal surface disorders. Furthermore an increased levels of matrix metalloproteinase-9 after this corneal surgery may be a second reason for corneal perforation after AMT.[32] Our patient had a deep sequestrum without any vascularization and this could explain the risk of corneal thinning and of increased level of metalloproteinases.

In conclusion, lamellar keratectomy and AMT could be an effective treatment for feline corneal sequestrum, with increased animal comfort and good corneal

transparency. A selection of the patients should be done, particularly for the very deep and not vascularized sequestra.

We also had really good results in the canine patients, without complications. There is no report in literature about the use of AMT after dermoid removal. In our two cases we had good cosmetic and functional results. More dermoid cases are needed to compare this technique to the traditional simple excision or the use of conjunctival graft.

Our results of the AMT in horses are similar to the data in the international literature. In our cases we used the inlay technique, and the amnion was placed with the basal membrane side up in those lesions where the integration of the AM is needed (melting ulcers, keratectomy bed after SCC excision or edema). In the fungal ulcers the amniotic basal membrane was placed side down. In these lesions the objective was, not only to have benefit from AM properties, but also to induce the migration of the residual fungi on the amnion. Even if it is not published yet, this technique is used by several colleagues. The fungi, indeed, are attracted by the glycoproteins of the amnion basal membrane.[36,37] The subsequent rejection of the amnion in these cases would consent the removal of the fungi.

We had complications in two patients. Both cases were affected by severe, deep fungal ulcer. The first horse developed an endothelitis one month after surgery; the second one had a corneal perforation under the amnion. These complications could be caused by the severe fungal infection and the impossibility to completely remove the fungi, with consequent progression of the lesion and collagenolisis.

In the other equine patients we had good cosmetic and functional results. We can conclude that AMT is an excellent alternative to the use of conjunctival graft or other surgical techniques that usually induce the formation of severe corneal scars, with visual deficits.

CHAPTER 6

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6. References

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CHAPTER 7

Acknowledgments

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