Canine Amniotic Membrane Transplantation for Ocular Surface Reconstruction of Created Deep Corneal Ulcers in Dogs

Simon Vongsakul1  Pranee Tuntivanich2  Sudson Sirivaidyapong3  Marissak Kalpravidh2*

Abstract

Canine amniotic membrane (AM) transplantation in conjunction with a third eyelid flap was used to promote healing of created deep corneal ulcers in 6 normal mongrel dogs. The healing was compared with the healing of created ulcers in the other eye of the same dog that were treated only with the third eyelid flap. A minimum of 60 days interval was allowed between the two treatment procedures in the 2 eyes of each dog. To simulate corneal ulcers found clinically, the surgical treatment was performed three days after the ulcer had been created. Cultures from preoperative conjunctival swabs revealed Staphylococcus spp., Streptococcus spp., and Enterococcus spp. The average time ± SE to complete corneal epithelialization in the eyes receiving the AM transplantation in conjunction with the third eyelid flap was 7.33±0.21 days which was significantly (p<0.05) shorter than the average time of 9.17±0.31 days observed in the eyes receiving only the third eyelid flap. Normal corneal transparency resumed in the eye receiving the AM transplantation and the third eyelid flap significantly (p<0.05) later than in the eye receiving only the third eyelid flap. Inflammation, neovascularization, and scar formation on the cornea, photophobia and impaired vision were not evident in any eyes at 8 weeks after either of the two surgical techniques. In conclusion, canine AM can promote the healing of created deep corneal ulcers in dogs.

Keywords: allograft, amniotic membrane, cornea, dog, transplantation, ulcer

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Introduction

Corneal ulceration is a common ocular disease that can lead to impaired vision in humans and animals. Primary causes of corneal ulcers are trauma, abnormal tear production, physiological imbalance, the abnormal development and function of the eyelids, and bacterial, viral and fungal infections. Prolonged inflammation of the cornea is detrimental to the corneal stem cells and the epithelial basement membrane resulting in neovascularization, corneal scarring and impaired vision (Thoft et al., 1979; Tsai and Tseng, 1995). In addition, neutrophils, keratocytes and abnormal epithelial cells produce collagenases and other proteolytic enzymes (Kenyon et al., 1979; Gilger, 2007) that can cause progressive ulceration of the corneal stroma with the risk of perforation. Perforation of the cornea potentially deteriorates to the eye. Continuous leakage of aqueous humor can result in a shallowing of the anterior chamber that leads to the anterior synechiae, glaucoma, cataracts, endophthalmitis or loss of vision. Therefore, prompt and appropriate treatment of corneal ulcers is extremely important.

The principles of corneal ulcer treatment include removal of the primary cause, reduction of inflammation, control of infection, enhancement of corneal healing and minimization of corneal scarring. Healing of the ulcer can be promoted surgically by the use of tissue adhesives and a soft contact lens, suturing, conjunctival flaps and grafts (Severin, 1996; Crispin, 2005; Gilger, 2007; Maggs, 2008). Tissue grafts used in reconstructing the ocular surfaces in animals are conjunctiva (Hakanson and Merideth, 1987), equine pericardium (Barros et al., 1999), egg shell membrane (Briksawan et al., 1999), canine peritoneum (Barros and Safatle, 2000), equine renal capsule (Andrade et al., 1999; Andrade et al., 2004), porcine small intestinal submucosa (Lewin, 1999; Featherstone et al., 2001;
Briksawan et al., 2003; Bussieres et al., 2004), human amniotic membrane (Kim and Tseng, 1995; Wichayacoop et al., 2005; Tuntivanich and Tuntivanich, 2006), equine amniotic membrane (Barros et al., 1998; Lassaline et al., 2005; Ollivier et al., 2006) and canine amniotic membrane (Barros et al., 2005).

Use of human amniotic membrane (AM) transplantation in the ocular surface reconstruction of the epithelial defects in human patients was proposed by Lee and Tseng in 1997. Since then, there have been several reports on the use of human AM transplants in various corneal disorders including burnt corneas (Shimazaki et al., 1997; Meller et al., 2000), limbal stem cell deficiency (Tseng et al., 1998), bullous keratopathy (Pires, et al., 1999), deep corneal ulcers (Kruse et al., 1999; Prabhasawat et al., 2001a; Solomon et al., 2002; Hick et al., 2005), neurotrophic corneal ulcers (Chen et al., 2000), descemetoceles and corneal perforations (Su and Lin, 2000; Duchesne et al., 2001; Hanada et al., 2001; Ma et al., 2002; Solomon et al., 2002; Rodriguez-Ares et al., 2004; Hick et al., 2005), infectious corneal ulcers (Kim et al., 2001), and defects after the excision of ocular surface neoplasias (Espana et al., 2002). These studies have shown that human AM transplants enhance epithelialization, reduce inflammation and vascularization and minimize scar formation on the cornea.

Like human AM, canine AM is the innermost layer of the placenta which consists of an epithelium, a thick basement membrane and stroma. From their similarities, canine AM might have properties comparable to those of human AM for the use in ocular surface reconstruction. Use of canine AM transplantation with favorable results has been reported in a dog with generalized keratomalacia, a cat with ankyloblepharon and a dog with corneal mass (Barros et al., 2005). The purpose of this study was to assess the healing of created deep corneal ulcers after canine AM transplantation in conjunction with the third eyelid flap in comparison with the healing after only the third eyelid flap in dogs.

**Materials and Methods**

**Animals:** The experimental animals were 6 healthy mongrel dogs, 2 males and 4 females. Their age ranged from 2 to 7 years and their weight from 11 to 17 kg. The study protocol has been approved by the Ethic Committee on Animal Use and Care of the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

**Preparation of canine AM:** Preparation of canine AM followed, with slight modifications, the method described by Lee and Tseng (1997) in preparing human AM. The least vascularized part of canine AM, the innermost layer of the placenta, was separated from the placenta obtained shortly after elective cesarean delivery. The AM was aseptically cleaned of blood clots with sterile phosphate buffer saline solution containing 0.1 mg/ml of gentamicin (Gentamicin Gibco®, USA) and 2.5 mcg/ml of amphotericin B (Fungisone Gibco®, USA). The AM was flattened onto a nitrocellulose paper with a pore size of 0.45 micron, with the epithelium/basement membrane side up. The paper with the adherent AM was cut into approximately 3x4-cm pieces and then preserved in a sterile container filled with storage medium. The storage medium was composed of equal parts (1:1, v/v) of glycerol and Dulbecco’s modified eagle medium (D-MEM Gibco®, USA), 0.1 mg/ml of gentamicin and 2.5 mcg/ml of amphotericin B. The preserved AM was stored at -70°C until used no later than 60 days.

**Surgical procedures:** Physical examination, complete blood count and blood chemistry evaluation were made one week before surgery. Food and water were withheld for a minimum of 6 hours before surgery. A drop of 1% tropicamide (Mydriacyl®, Alcon, Belgium), 0.5% ketorolac tromethamine (Acular®, Allergan, Ireland), and 0.3% tobramycin (Tobrex®, Alcon, Belgium) were applied 3 times at 15 minutes interval before the operation. Dogs received 10 mg of chlorpheniramine subcutaneously 30 min before the intramuscular premedication with 0.04 mg/kg atropine sulfate and 0.2 mg/kg acepromazine maleate. Anesthesia was induced with thiopental sodium and maintained with halothane in oxygen.
The dog was placed in a lateral recumbency and the eye to be operated on was prepared for surgery. The corneal ulcer was created in both eyes of each dog with a minimum of 60 days interval between the two treatment procedures. An ulcer was created deep in the stromal layer at the central cornea by using a 5 mm trephine and a scalpel blade. All ulcers were created by the same surgeon in order to control a consistent depth of the ulcer. To simulate the clinical corneal ulcers, all created ulcers were treated three days after they had been created and the conjunctival sac was swabbed for bacterial cultures before surgery. The two eyes of each dog were randomly selected to receive the two different treatment procedures. The ulcer in one eye was treated with the third eyelid flap sutured to the bulbar conjunctiva while the ulcer in the other eye was treated with canine AM transplantation and the third eyelid flap. To apply the canine AM on the created ulcer, an individual piece of the nitrocellulose paper with the adherent AM was thawed, removed from the storage media and rinsed with sterile saline to remove any remaining glycerol in which it had been stored. The paper with the AM was cut to a size a little larger than the ulcer width. The AM was peeled from the paper and laid with the stromal side facing the ulcer. The AM graft was secured to the healthy cornea, 3 mm from the edge of the ulcer, by simple interrupted 10/0 nylon sutures and then covered by the third eyelid flap sutured to the bulbar conjunctiva with simple interrupted 6/0 silk.

After surgery, the dogs received 4 mg/kg gentamicin sulfate and 0.4 mg/kg dexamethasone subconjunctivally before recovery from anesthesia, 25 mg/kg ceftriaxone and 0.2-0.5 mg/kg dexamethasone intramuscularly, and 200,000 IU vitamin A subcutaneously for 7 days. After eyewash once a day with 2% boric acid, topical medications were 16% gentamicin sulfate and artificial tears (Systane®, Alcon, USA) 4 times a day, and 1% atropine sulfate (1% Isopto atropine®, Alcon, Belgium) 2 times a day. The dogs were followed-up daily during the first 2 weeks and weekly thereafter. After stitches had been removed from the third eyelid 7 days after the operation, the cornea was stained with fluorescein and examined under biomicroscope for the persistence of the ulcer and the AM graft. The stitches on the cornea were removed on day 10 after surgery. Photophobia, inflammation, neovascularization and opacity of the cornea were also observed. The persistent ulcer was treated by instillation of 16% gentamicin sulfate and artificial tears 4 times a day and 1% atropine sulfate 2 times a day until healing of the corneal epithelium had been completed, confirmed by a negative fluorescein retention. Then a solution of 0.1% dexamethasone, neomycin sulfate and polymyxin B sulfate (Maxitrol®, Alcon, Belgium) was topically administered 4 times a day for 7 days after which topical 0.1% fluorometholone acetate (Flarex®, Alcon, Belgium) was used 4 times a day.

**Analysis:** The time to complete corneal epithelialization, confirmed by a negative fluorescein retention at the corneal lesion, and the time to attain normal corneal transparency, confirmed by disappearance of the corneal opacity, in the eyes receiving different treatment procedures in each dog were compared by paired t-test at \( p < 0.05 \).

**Results**

**Cultures from conjunctival swabs:** Cultures from conjunctival swabs of 12 eyes revealed *Staphylococcus* spp. in 8 eyes, *Streptococcus* spp. in 2 eyes, *Streptococcus* spp. and *Enterococcus* spp. in 1 eye, and no bacterial growth in 1 eye (Table 1).

**Eyes receiving only the third eyelid flap:** Observation of the eyes before the third eyelid flap application revealed keratitis with slight superficial neovascularization in all 6 eyes. Eye discharge was noticed in all eyes two days after the third eyelid flap. Examination of the eye after the removal of stitches from the third eyelids 7 days after surgery revealed no evidence of keratitis and neovascularization that had been found previously. Complete epithelialization of the cornea was found on day 8, 9 and 10 after surgery in 1, 3 and 2 eyes, respectively.

The average time ±SE to complete epithelialization of the
cornea following the third eyelid flap was 9.17±0.31 days (table 1). Corneal opacity completely subsided and normal transparency of the cornea resumed at week 5, 6 and 7 after surgery in 1, 4 and 1 eyes, respectively. The average time ±SE that normal transparency resumed in all 6 corneas was 6±0.26 weeks. Neovascularization, opacity and scar formation on the cornea, photophobia and impaired vision were not found in any dogs at 8 weeks after surgery.

**Eyes receiving amniotic membrane transplantation and the third eyelid flap:** As observed in the other 6 eyes receiving only the third eyelid flap, keratitis with slight superficial neovascularization was found preoperatively in all 6 eyes on the day of AM transplantation. Eye discharge was observed in all 6 eyes two days after the transplantation. Following the removal of stitches from the third eyelids 7 days after the transplantation, examination of the eyes revealed that keratitis and neovascularization found previously subsided. Canine AM completely disappeared from 4 eyes but still partially attached at the

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Microorganisms</th>
<th>3rd eyelid flap (one eye)</th>
<th>Canine AM/ 3rd eyelid flap (another eye)</th>
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<td>1</td>
<td><em>Streptococcus</em> spp. and <em>Enterococcus</em> spp. (right eye); no growth (left eye)</td>
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<td>7</td>
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<td>2</td>
<td><em>Staphylococcus</em> spp. (both eyes)</td>
<td>8</td>
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<td>3</td>
<td><em>Staphylococcus</em> spp. (both eyes)</td>
<td>10</td>
<td>7</td>
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<tr>
<td>4</td>
<td><em>Streptococcus</em> spp. (right eye)</td>
<td>9</td>
<td>7</td>
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<tr>
<td>5</td>
<td><em>Staphylococcus</em> spp. (right eye)</td>
<td>9</td>
<td>7</td>
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<tr>
<td>6</td>
<td><em>Staphylococcus</em> spp. (both eyes)</td>
<td>10</td>
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<td>Average±S.E</td>
<td>9.17±0.31</td>
<td>6±0.26</td>
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* : significantly different (p<0.05) from the respective times observed in the eyes receiving only the third eyelid flap

**Table 1** Microorganisms identified from conjunctival swabs from 12 eyes before treatment, the time to complete epithelialization of the cornea, and the time for corneal opacity to subside after the third eyelid flap and transplantation of canine amniotic membrane in conjunction with the third eyelid flap for ocular surface reconstruction of created deep corneal ulcers in 6 mongrel dogs.
**Discussion**

*Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp. found in the preoperative conjunctival swabs 3 days after creation of the ulcer can be found in the normal eyes of dogs. However, they are the opportunistic bacteria that can make ocular infection more severe (Moore and Nasisse, 1999). They produce a number of enzymes and enterotoxin that enhance the progression of the infection and the destruction of the cornea. In this study, only mild inflammation with slight neovascularization on the cornea was observed 3 days after the ulcer had been created. The ulcer might not well mimic clinical deep corneal ulcer and not require AM transplantation. This may be the reason why there is no marked difference between the time to resume normal corneal transparency in the eye receiving only the third eyelid flap and the time in the eye receiving AM transplantation and the third eyelid flap. However, complete corneal epithelialization was more rapid in the eye receiving AM transplantation. The rapid epithelialization of the cornea is very important for ocular surface reconstruction. The more rapidly the ulcer heals, the less the scar formation. Corneal ulceration will progress if exposure to the tear film and microorganisms and inflammation of the stroma are prolonged. Collagenases and other proteolytic enzymes produced by neutrophils in the tear film, abnormal epithelial cells, keratocytes or fibroblasts, and microorganisms will cause marked degradation of collagen in the stroma resulting in a melting cornea (Gilger, 2007).

In orienting an AM graft against the recipient cornea, the graft will be incorporated if its stromal side faces the cornea; but it will slough off if its basement membrane faces the cornea (John, 2003). When the stromal side faces the cornea, the AM acts as a graft to facilitate epithelial cell migration, reinforces adhesion of the basal epithelium, promotes cellular differentiation and prevents apoptosis (Rodriguez-Ares et al., 2004). When the basement membrane faces the cornea, the AM acts as a barrier in protecting epithelial growth from eyelid rubbing and preventing tear inflammatory cells and proteins from contacting the corneal stroma. Regenerating corneal epithelium grows along the basement membrane to fill the defect.

In placing the AM over the cornea, if AM is large and laid covering beyond the edge of the ulcer or the entire cornea, it will be called “overlay or AM graft or path” (Hanada et al., 2001; Letko et al., 2001; Solomon et al., 2002; Rodriguez-Ares et al., 2004). If AM is placed with its shape and size fit to the corneal ulcer, it will be called “inlay graft or AM filling” (Hanada et al., 2001; Letko et al., 2001). The present study applied canine AM as an overlay graft of the size a little larger than the ulcer with its stromal side facing the recipient cornea. Part of the AM contacting the ulcer was incorporated with the recipient corneal stroma while the AM part contacting the normal cornea sloughed off. Some studies found human AM disintegrated or dissolved (Lee and Tseng, 1997; Kruse et al., 1999; Letko et al., 2001; Sridhar et al., 2001; Solomon et al., 2002; Rodriguez-Ares, 2004).

Epithelialization of the cornea in the eye receiving AM transplantation followed by the third eyelid flap was completed earlier than that observed in the eye receiving only the third eyelid flap. Like a contact lens used in human patients, the third eyelid flap provides mechanical protection to the healing cornea from trauma and lid rubbing (Gilger, 2007). The time to complete corneal epithelialization of 7.33±0.21 days after the use of canine AM graft in conjunction with the third eyelid flap in this study was comparable to the time after the use of human AM graft and the third eyelid flap in a similar ulcer model in 5 dogs (Wichayacoop et al., 2005). This suggests that canine AM may contain factors similar to those of human AM. Human AM contains a number of growth factors (Koizumi et al., 2000) that favor epithelial healing while its basement membrane facilitates the migration of epithelial cells and reestablishes adhesion between new epithelial cells and the underlying basement membrane (Khodadoust et al., 1968). In addition, the stromal matrix of the membrane contains proteinase inhibitors that promote an efficient epithelial healing and reduce stromal
inflammation of the cornea (Kim et al., 2000). Shimazaki et al. (1998) claimed that human AM epithelium containing growth factors may survive up to 70 days after cryopreservation. In this study, the factors within the canine AM should remain because the membrane was used no later than 60 days after preservation.

Neovascularization and corneal scarring were not found at 8 weeks after surgery in any eyes. The created corneal ulcers might not be severely traumatized and did not need AM transplantation. This might be the reason why the ulcers receiving only the third eye lid flap were healed with no neovascularization and corneal scarring at 8 weeks after surgery. Canine AM may have properties similar to those of human AM which is avascular and strong and has anti-inflammatory, anti-angiogenic (Hao et al., 2000) and anti-scarring effects (Tseng et al., 1999). These qualities make human AM a good substrate for reconstructing damaged surface of the cornea (Lee and Tseng, 1997; Shimazaki et al., 1997; Tseng et al., 1998; Pires et al., 1999) and the conjunctiva (Tseng et al., 1997). Several factors contribute to the anti-inflammatory action of human AM such as precluding polymorphonuclear cell infiltration (Park and Tseng, 2000). The membrane provides an effective barrier against tear film. This significantly reduces the number of inflammatory cells that invade the stromal ulcer via the tear film. Therefore, the number of inflammatory mediators that cause the inflammatory response is reduced. Human AM also contains certain inhibitors of proteinases that contribute to corneal melting (Kim et al., 2000) and that additionally may down-regulate the synthesis of chemokines in keratocytes in response to inflammatory mediators (Kruse et al., 1999). Both effects favor a reduction in inflammation, neovascularization, and fibrosis or scarring (Hao et al., 2000; Tseng et al., 1999). The anti-scarring effect may be the result of the anti-inflammatory mechanism as well as the prevention of fibroblast activation into myofibroblasts (Tseng et al., 1999) and by the stromal matrix of the AM that reduces the keratocyte apoptosis (Wang et al., 2001) or traps the inflammatory cells (Shimmura et al., 2001). An anti-angiogenic effect could be a function of the inhibitors of metalloproteinases in the AM (Hao et al., 2000).

Inflammation of the trephined cornea caused edema resulting in temporary corneal opacity. However, the opacity was minimal and did not impair the vision of the dogs. Suturing the AM graft with the cornea may increase inflammation of the cornea. This might be the reason why normal transparency resumed in the corneas receiving the AM transplantation in conjunction with the third eyelid flap later than in the corneas receiving only the third eyelid flap. To minimize opacity and the time taken to return normal transparency of the cornea, the AM graft is better sutured with the bulbar conjunctiva instead of the cornea. No allograft rejection was found in this study. The postoperative steroid administration may suppress the rejection and the AM when preserved does not contain live cells to initiate the immune reaction (Lee and Tseng, 1997; Tseng et al., 1999; Kruse et al., 1999).

Transplantation of human AM for ocular surface reconstruction is mostly performed to repair the corneal ulcers that are large and/or deep, involving 50% or more of corneal thickness; ulcers that are perforated and also ulcers refractory to conventional treatment (Solomon et al., 2002; Gilger, 2007). The transplantation commonly uses multilayer membrane (Prabhasawat et al., 2001b; Solomon et al., 2002; Rodriguez-Ares et al., 2004) and the number of AM layers to be used depends on the depth of the ulcer. The deeper the perforation size, the greater the number of AM layers is required. Normally, the innermost and subsequent layers of a size and shape to fit the corneal defects are placed as inlay grafts while the outermost layer is applied as an overlay graft covering the entire cornea. The number of AM layers used affects the time to complete corneal epithelialization. Rodriguez-Ares et al.(2004) reported a mean epithelialization time ranging from 2 to 6 weeks after using 2-4 layers of human AM in 15 human eyes with corneal perforations of different sizes. They found that the time to complete the epithelialization of the perforations increased with
increasing layers of the AM. The use of a single layer of canine AM and the minimal extent of the corneal damage in our dog model might have contributed to the short epithelialization time.

**Conclusion**

Canine AM provides rapid epithelialization without neovascularization and scarring of the cornea which is important for the healing of ocular surface disorders.

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**References**


