BOVINE AMNIOTIC MEMBRANE DERIVED EXTRACELLULAR MATRIX SCAFFOLD FOR CORNEAL WOUND HEALING IN RABBIT MODEL

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(21-MVP-21)

THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Bovine amniotic membrane derived extracellular matrix scaffold for corneal wound healing in rabbit model"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship, or other similar title, of any other University or society.

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EXTERNAL EXAMINER

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1. INTRODUCTION

Corneal ulcers are challenging ophthalmic emergencies that are still raising threats to patients globally. The healing process during corneal ulcerations is prolonged which elevates the total morbid period and decreases the quality and productivity of life. There is a necessity to accelerate the healing and return the affected cornea to its normal function. To accomplish accelerated healing and early return of normal corneal function, several treatment strategies have been studied, in which different kinds of biological membranes as corneal grafts are proposed for reconstructive ophthalmic surgery. The various biological membranes used as grafts in veterinary practice include equine, canine and human amniotic membranes, equine or bovine pericardium, porcine small intestinal submucosa, porcine urinary bladder acellular matrix, buccal mucous membrane, and autologous platelet-rich fibrin membrane. Among the biological membranes studied, amniotic membranes have demonstrated outstanding outcomes.

Amnion is the innermost layer of the foetal membrane. Structurally, amniotic membrane includes layers such as the epithelium, basement membrane and stroma which is reported to facilitate the migration of corneal epithelial cells when grafted on cornea. Studies have proved that amniotic membranes when used as a graft to treat corneal lesions, reduced vascularisation and scarring of the cornea during healing. They promote epithelial cell migration, adhesion, and proliferation forming a better structural framework for the healing process. They are better known to suppress the expression of certain inflammatory cytokines as well as express several growth factors that aid in rapid healing of cornea.

Bovine amniotic membrane (BAM) is an easily available membrane for wound healing purpose in veterinary practice. Bovine amniotic membrane can be collected from normal vaginal delivery or caesarean section. They can be decellularised to make it less immunogenic and avoid graft rejection. Large production of decellularized BAM can be achieved even from a single collection and could be a cost-effective alternative for wound-healing.

Preclinical studies of this membrane for corneal wound healing is lacking. Histological studies are required for choosing decellularised bovine amniotic membrane as a scaffold corneal wound healing. The BAM derived ECM scaffold could be an excellent graft material because it has been earlier reported that amniotic membranes has anti-inflammatory, anti-angiogenic, anti-scarring, anti-fibroblastic, and antimicrobial properties and act as a framework to support epithelial cell migrations promoting faster tissue regeneration. Rabbits remain as a popular choice for experimental ophthalmology research primarily due to their larger size of the eyes in proportion to their body as well as their close resemblance with human, canine, and feline eyes. Hence the present study is undertaken to evaluate the efficacy of bovine amniotic membrane-derived extracellular matrix as a scaffold for corneal wound healing in rabbit model.

2. REVIEW OF LITERATURE

2.1. ANATOMY OF RABBIT EYE AND CORNEA

According to Davis (1929), the rabbit cornea is unusually prominent and wide. Its radius of curvature measured about 7.3 mm, with its shape roughly elliptical and having a longer horizontal axis. The horizontal diameter averaged about 15.6 mm., and the vertical about 13.8 mm. The thickness of the cornea was fairly uniform, being slightly greater at the limbus. Thickness averaged 0.37 mm at the centre and 0.45 mm near the limbus.

Maurice (1995) stated that the orbital glands, including the large Harderian gland, produced tears, which explains the exceptionally slow blink rate - one or two blinks per minute in rabbits. According to the authors, the exceptionally slow blink rate has deleterious consequences on corneal epithelial healing and drug delivery.

Beurman and Pedroza (1996) reported that Descemet's membrane is the thick basement membrane of the corneal endothelium and is very rich in collagen. Authors also reported that corneal endothelium comprised of an active metabolic pump which helps in maintaining corneal clarity.

Ojeda *et al*. (2001), through scanning electron microscopic (SEM) studies, documented that corneal epithelium appeared smooth, on which the cell boundaries could not be resolved. The epithelial surface was randomly infiltrated by numerous nerve fibers. The authors also reported that the rabbit epithelial basement membrane (EBM) appeared as a flat network of straight fibers with no defined order. also, the stroma comprised stromal lamellae, keratocytes, and stromal neural plexus.

Andrew (2002) stated that 30% of the eyball in rabbits comprised of cornea, with dimensions of approximately 15 mm horizontally and 14 mm vertically. The authors also reported that the layers of the cornea from outer to inner, comprised of the epithelium, which is a keratinized layer 30-40 micrometers thick, then followed by the stroma which is the thickest layer, made up of collagen bundles arranged

parallelly, followed by Descemet's membrane which is 7-8 micrometers thick and continuously getting thickened with age and reaching 15 micrometers thick in aged rabbits, and finally the inner endothelium, which is single cell layer containing crucial ATP-ase pump that played a major role in maintaining corneal transparency.

Klenker *et al*. (2007) opined that corneal epithelium is protected by an anterior tear film, which prevents damage and entry of pathogens and contributes to growth factors like epidermal growth factor (EGF), which promotes epithelial regeneration.

According to Samuelson (2013), the third eyelid lacked muscles but was passively drawn more than two-thirds of the way across the eyeball by the pull of the powerful retractor oculi muscles. The lack of an orbital fat pad rendered the rest of the orbit filled only by the retrobulbar muscles, the orbital glands, and the globe itself. Tear drainage is through a single slit-like opening 3–4 mm from the eyelid margin. There was no defined lacrimal sac as in the dog, cat, and human, but rather an elongated dilatation of the nasolacrimal duct.

Bukowiecki *et al*. (2017) classified layers of cornea as anterior epithelial layer, epithelial basement membrane, stromal layer, Descemet's membrane, and an endothelial cell layer. The authors reported that this stromal layer comprised of tightly packed collagen fibers and sparsely spread keratocytes. The Descemets membrane constitutes the basement membrane for the endothelium. The endothelial cell layer served as the innermost portion of the cornea and was in direct contact with aqueous humor.

2.2. PHYSIOLOGY OF RABBIT CORNEA

Hughes (1972) stated that rabbits, as a prey species, possessed a visual system that concentrated on the horizon. This allowed them to gather visual input for almost 360 degrees of circumference providing an almost complete visual field.

Klyce and Crosson (1985) opined that corneal epithelium helped in corneal hydration, but its fluid-transporting ability was many times less than that of

endothelium. The endothelial pump normally functions at some fixed capacity, but activity becomes questionable when there was an underlying pathology or ageing.

Bukowiecki *et al*. (2017) mentioned that the cornea lacked blood and lymphatic vessels. The cornea receives its oxygen and nutrients through tear film on its outer side, aqueous humor on its inner side, and limbal vasculature on its periphery.

2.3. RABBIT AS AN EXPERIMENTAL MODEL

Rabbits have been used as a model for studying radiation-induced cataracts since the 1950s. Investigators performing radiation studies in rabbits observed that cataracts developed similar in structure to radiation-induced cataracts in humans. (Cogan and Donaldson, 1951).

Gimborne *et al.* (1974) studied prevascular tumor growth and the host's angiogenic response to live tumor cells and tumor-angiogenesis factors-3, 6 in rabbit cornea. Implantation at varying distances from the circumferential limbal vessels produced an anatomic separation of tumor cells from responding host vessels, allowing independent observations of both elements' behavior.

Myles *et al*. (2005) reported that rabbits, considering the size of their eyes, are one of the most widely used species, to investigate novel methods of ocular drug delivery to the posterior segment for treatment of vitreoretinal diseases.

The significant size of the rabbit eye relative to its body size makes them a desirable and cost-effective choice for conducting surgical and drug delivery experiments. The eyes of rabbits shared few similarities with human eyes. The rabbits have thinner cornea compared to humans and a larger lens, causing the iris to bulge forward, creating a more curved anterior chamber (Werner *et al*., 2006).

2.4. INDUCED CORNEAL WOUNDS

Jester *et al*. (1992) employed radial nonpenetrating corneal incisions for studying corneal healing in rabbits and cats. Incisions were made with the help of a diamond knife with its micrometer adjusted to 85 percent of the central corneal

thickness as determined by ultrasonic pachymetry. A three mm central optical zone centered above the pupil was demarcated using an optical zone marker. Radial incisions started at the central optical zone and extended peripherally to 2-3 mm inside the limbus.

Stepp *et al*. (1993), in their review on corneal wound healing studies, reported that the typical wound size in rabbits is 5 or 6 mm while it is 3 mm in rats. The authors also reported that Wounds were created with the help of trephines of different diameters to demarcate the area within which tissue is removed. Sharp trephines penetrated tissues easily. The trephines were dulled using Arkansas stone or similar tools to prevent them from penetrating the stroma.

Wang *et al*. (2001) performed transepithelial ablation using the VISX Star laser under a phototherapeutic keratectomy (PTK) mode using an ablation diameter of 6.0 mm and a depth of 120 mm without edge smoothing for their studies in rabbit corneas. In their study, one eye was used as a test while the other served as a control. The keratectomy wounds were covered with a preserved human amniotic membrane of 9 mm diameter.

Li *et al*. (2016) mechanically debrided the limbus in New Zealand white rabbits using Algar Brush II rotating burr for creating and studying limbal stem cell deficiency. The debridement was done in such a way that the burr tip was applied with light pressure and in a circular motion starting at the 12 o'clock position of the eye and slowly moving around the entire periphery of the cornea.

Capistrano da Silva *et al*. (2021) experimented with bovine amniotic membrane homogenate for healing studies on ex-vivo corneas in rabbits. A 6 mm diameter and 0.125 mm depth circular keratectomy was made using a Hessburg-Barron vacuum corneal trephine and crescent knife in the central cornea. Following creating a corneal wound, corneoscleral rims were aseptically harvested by incising circumferentially approximately 7 mm posterior and parallel to the limbus.

2.5. BOVINE AMNIOTIC MEMBRANE

Schwarze (1972) studied the amniotic membrane in ruminants and reported that the lining of the amniotic cavity in ruminants was clear and lacked significant blood supply while they had extensive connections with the chorion. There were abundant glycogen-rich villi or plaques in the amniotic membrane, which formed around the third month of gestation.

Lee and Tseng (1997) noticed that the amniotic membrane served as a platform for epithelial cell growth and axonal regeneration. The authors also reported that the amniotic membrane supports epithelialisation by aiding the migration of epithelial cells, strengthening their adhesion to the underlying layer, promoting differentiation, and preventing cell death through its anti-apoptotic properties.

Tseng *et al.* (1999) stated that amniotic membrane has anti-inflammatory properties through down-regulation of TGF-beta signaling systems. The authors reported the presence of IL-1 receptor antagonists and IL-10 in the amniotic membrane, potent inflammation inhibitors. The authors studied and reported that the anti-scarring effects of the amniotic membrane were due to the prevention of fibroblast activation into myofibroblast.

Hao *et al*. (2000) reported that fresh and preserved amniotic membranes have anti-angiogenic effects, and these effects were enhanced by chemicals such as thrombospondin-1, endostatin, and inhibitors of metalloproteases.

Koizumi *et al*. (2000) reported the presence of growth factors such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and transforming growth factors β (TGF-β) in the bovine amniotic membrane.

Schlafer *et al*. (2000) stated that the bovine fetal membrane consists of the chorion, the allantois, and the amnion. During development, the amniotic

membrane partially fuses with the chorion, forming the chorioamnion, and partially with the allantois forming the allantochorion. The authors also stated that the amnion typically appears white and smooth apart from chorion or allantois which is red or velvety.

Amniotic membrane or amnion is a highly suitable option for grafting due to its pluripotent cell properties. Serving as the innermost layer of the placenta, it lacked blood vessels and performed various roles such as the transport of water and soluble materials and the production of bioactive factors, including vasoactive peptides, growth factors, and cytokines (Toda *et al*. 2007).

Niknejad *et al*. (2008) observed that the extracellular matrix of the amniotic membrane consisted of collagen I, III, IV, V, VI, and XV, along with laminin, nidogen, fibronectin, and proteoglycans. The authors reported the presence of these collagen in extracellular matrix structure hold promise for amniotic membrane as a biomedical material for tissue engineering.

Park *et al*. (2008) reported that. histologically amniotic membrane comprised of a single layer of cuboidal epithelium. The authors also documented that acellular bovine amniotic membrane proved to be a reliable and efficient dressing material. Bovine amniotic membrane enhanced wound healing capabilities and acted as a barrier against bacterial infection. Massive production and long-term storage were possible and were easy to apply.

Kim *et al*. (2009) reported promising efficacy with bovine freeze-dried amniotic membrane in the healing of corneal epithelial wounds in dogs. The authors also reported that bovine freeze-dried amniotic membrane would be a viable alternative treatment for canine superficial corneal ulceration, surpassing conventional therapies.

Mamede *et al*. (2012) observed histologically that, the amnion is composed of epithelial cells, an acellular basement membrane, and mesenchymal cells. The layer that was adjacent to the amniotic fluid was the inner layer, which was composed of a single homogeneous layer of cuboidal epithelial cells firmly attached

to the basement membrane. The basement membrane was comprised of abundant heparan sulfate, acting as a permeable barrier to macromolecules and was attached to a condensed acellular layer of collagens type I, II, and V.

Litwiniuk and Grzela (2014) opined that corneal ulcers and other ocular disorders, such as perforation, chemical or thermal burns, keratomalacia, etc, could be treated by an amniotic membrane. Authors stated that Amniotic membrane (AM) has been popular because of its biocompatibility, anti-proteolytic, anti-angiogenic, anti-inflammatory, antifibrotic, anti-scarring, antibacterial, and reepithelializing properties.

Min *et al*. (2014) reported better aesthetic results and healing after applying BAM for wound repair on the skin surface. Authors reported that histologically amniotic membrane consists of cuboidal as well as flattened epithelial cells and mesenchymal connective tissue. Epithelial cells migrate and form a leading edge over the wound surface. The mesenchymal connective tissue contained laminin, fibronectin, and various types of collagen. The authors opined that the amniotic membrane provided physical coverage over the wound bed. Processed amniotic membrane shared structural similarities with dermal basal membrane, enabling epithelial keratocytes to migrate effectively through the scaffold.

Pontes *et al.* (2014) compared and studied outcomes of penetrating keratoplasty using bovine amniotic membrane and n-butyl 2- cyanoacrylate. The authors noticed that in the groups that received the amniotic membrane alone, the amniotic membrane acted as a scaffold and aided in superior repair. Histological evidence confirms the migration of endothelial cells on the amniotic membrane. The authors also proposed that the combination of amniotic membrane along with tissue adhesive provided greater corneal repair than using it alone.

Favaraon *et al*. (2015) reported that the amniotic membrane comprises three fundamental layers: the epithelial layer, the basal layer, and the mesenchymal layer, irrespective of the species. These layers play a vital role in maintaining structure and function of amniotic cells.

According to Stachon *et al.* (2015), the precise mechanism by which amniotic membrane enhanced corneal healing remains uncertain; studies indicated that it could be attributed to the interactions of cytokines, growth factors, and protease inhibitors.

Al-Falahi *et al.* (2017) compared the healing effects of bovine amniotic and bovine pericardial membranes in rabbit wounds. Authors opined that the amniotic membrane served as a biomaterial for wound healing; both bovine amniotic membrane, and pericardial membrane showed beneficial effects in the healing of rabbit wounds.

Capistrano da Silva *et al*. (2021) reported from their ex-vivo corneal healing studies in rabbits that the bovine amniotic membrane had a tremendous effect in speeding up the rate of corneal epithelial restoration. Authors emphasized that as a potent therapy to improve stromal and epithelial healing.

2.6. PROCESSING OF BOVINE AMNIOTIC MEMBRANE

Park *et al*. (2008) collected amniotic membranes from healthy placenta, disinfected using 70% ethanol and 0.05% sodium hypochlorite. The epidermis was removed, and the stromal layer was treated with 0.25% trypsin, 0.025 EDTA, and 0.9% NaCl at pH 7.4 for 60 minutes to remove the cellular components. The acellular AMs were dried to maintain their biochemical and structural properties. Individual AMs were sterilized with 25 kGy of gamma irradiation and stored below room temperature until further use.

Said *et al*. (2009) reported that after processing and preservation of the amniotic membrane, the epithelial and stromal cells in the amnion were rendered nonviable. The authors also reported that even though epithelial cells were visible in the thawed membrane, the stroma was particularly devoid of cells, and practically no DNA could be extracted from the amniotic stroma.

Al- Falahi *et al*. (2017) opined that sterile amniotic membranes obtained from post-partum cows can be decellularized in a mixed solution containing 0.1% acetic acid and 4% ethanol for 2 hours. The resulting materials were rinsed with

Phosphate Buffered Saline (PBS) and deionized water for 15 minutes. Further storage was done at 4 ^{0}C in PBS along with 1% gentamicin.

Ballosteros *et al*. (2020) reported that the bovine amniotic membrane could be decellularised by keeping the amniotic membrane in liquid nitrogen (-196 ^{0}C) for 22 hours, followed by a thawing cycle for 2 hours at 37 ^{0}C. Subsequently, they were treated with both strong and weak detergents, sodium dodecyl sulfate (SDS) or Tween 80 for 4 hours. This was followed by immersion in a base solution (0.1M NaOH) for 1 hour and then an acid solution (a combination of peracetic acid and ascorbic acid). As a final step, membranes were washed in 70 percent ethanol for 1 hour to remove residual nucleic acids and phospholipids, followed by a two-hour rinse with phosphate-buffered saline solution. Throughout the procedure, membranes were mechanically stirred in an orbital shaker to ensure thorough washing while minimizing damage to tissue ultrastructure.

Capistrano da Silva *et al*. (2021) studied rabbit ex-vivo corneal healing using bovine amniotic membrane homogenate. The Authors stated that the bovine amniotic membrane underwent three washes for a period of three hours with sterile phosphate buffered saline and an antibiotic-antimycotic solution. Subsequently, the membranes were aseptically cut into pieces and kept in a solution containing 50 microgram/mL gentamicin and Hank's balanced salt solution (HBSS). Until further use, the membranes were stored at -80 ^{0}C and were used after thawing at room temperature.

2.7. SURGICAL TECHNIQUE INVOLVING AMNIOTIC MEMBRANE

Kruse *et al*. (1999) reported the efficacy of multilayer amniotic membrane transplantation in addressing deep ulcers that were unresponsive to conventional treatments. The authors filled the defect with amniotic membrane pieces followed by overlaying them with an amniotic membrane and securing it to the cornea through suturing. The authors noted epithelialisation, reduction in ocular inflammation, and restoration of corneal stromal thickness.

Su and Lin (2000) treated corneal perforations with a combination of amniotic membrane and tissue adhesive. The authors inserted a 1.5 mm section of amniotic membrane directly under the perforation in the anterior chamber and applied cyanoacrylate adhesive over the perforation.

Letko *et al*. (2001) studied inlay and overlay approaches using amniotic membranes for treating persistent corneal defects. The authors noticed no disparity between the inlay and overlay techniques considering the recurrence rate of defects and duration taken for healing.

John *et al*. (2002) reported that toxic epidermal necrolysis could be successfully managed by grafting with a human amniotic membrane. The Authors surgically grafted sections of human amniotic membrane over the affected eyes. Membranes were fixed around the limbus of the eye using multiple 10-0 vicryl sutures.

Dua *et al*. (2004) stated that 10-0 nylon, as well as 8-0 to 10-0 vicryl, are the commonly used suture materials for securing the amniotic membrane over the cornea. Interrupted, running, or mattress types of sutures are the preferred suture patterns. Specifically, mattress sutures are placed tangentially to the limbus, securing it to the episclera or the superficial sclera.

Tseng *et al*. (2004) reported that amniotic membrane can be used for various ocular surface reconstruction procedures as a long-term or temporary solution. In permanent applications, surrounding host cells migrated into the amniotic membrane. In temporary applications, the amniotic membrane serves as a patch or dressing, allowing the host cells to migrate underneath it. After complete healing membrane dissolves naturally.

Non-healing corneal ulcers can be managed by the application of a single layer of amniotic membrane sutured over the ulcer to cover the defect. But in corneal thinning, descemetocele, and corneal perforations, multiple layers of amniotic membrane were used to fill the depth of the defect. Grafts were placed with their epithelial side facing up and sutured with 8-0 to 10-0 Vicyl or Nylon.

Chemical burns, spheroidal degeneration, bullous keratopathy, etc are managed by superficial keratectomy with amniotic membrane transplantation (Thatte, 2011).

2.8. CORNEAL HEALING

Kamiyama *et al*. (1998) reported that platelet-derived growth factor (PGDF) isoforms, including PDGF-AA, PDGF-BB, PDGF-AB, and particularly PDGF-AB, were found to promote the migration of rabbit corneal epithelial cells. The authors noticed this effect when fibronectin, which constituted a part of the provisional extracellular matrix, was found at the wound site.

Jester *et al*. (1999) reported that the corneal epithelium heals through-migration, mitosis, and differentiation. The authors described the course as follows: when the injury extends up to the corneal stroma, a healing process is initiated with apoptosis of keratocytes near the wound site, followed by activation, transformation, and migration of keratocytes from surrounding areas. These activated keratocytes adopt a fibroblast-like phenotype and begin secreting the extracellular matrix. Subsequently, some of the fibroblasts differentiated into myofibroblasts, which have contractile properties.

Kruse *et al*. (1999) opined that corneal perforation and stromal melting can be treated by filling the defects with multilayer of amniotic membrane transplants. The authors reported that the membrane served as a scaffold for keratocytes to migrate and rebuild stromal tissue effectively. The authors also noted that collagen arrangement in these regenerated areas differs from that of normal cornea.

Ma and Bazan (2000) noticed that PAF or platelet-activating factor, which is a bioactive lipid compound, appeared in the cornea following injury. The presence of PAF triggers various biochemical reactions linked to inflammation and the healing process. PAF quickly activated phospholipase A2, leading to the production of arachidonic acid and specific production of prostaglandins in the corneal epithelium. It further encouraged the production of COX-2, which synthesized prostaglandins associated with the inflammatory response.

In a study by Chandrasekhar *et al*. (2001), the authors reported that in organ-cultured rabbit corneas, keratinocyte growth factor (KGF) accelerated the healing of corneal epithelial wounds. Apart from this, KGF was found to boost cell proliferation in the limbal epithelium.

Multiple cytokines, including IL-1, TNF-α, bone morphogenetic proteins BMP-2 and 4, epidermal growth factor, and platelet-derived growth factor, are released from the injured corneal epithelium and epithelial basement membrane. These cytokines contributed to cell migration, cell proliferation, and cell adhesion, as documented by Wilson *et al*. (2001).

Gris *et al*. (2002) noticed that when amniotic membranes were used to correct stromal defects, the membrane initially filled the defect and later got substituted by fibroblasts and collagen.

Wollensack *et al*. (2007) reported from their studies on corneal collagen crosslinking with riboflavin and UV-A in rabbit models that epithelial closure occurred within 3-7 days. Authors noted mild inflammatory infiltrate consisting of polymorphonuclear leukocytes, lymphocytes, and macrophages. Stromal oedema resolved after one week, with keratocyte repopulation and mitoses. Endothelial layers were found to be intact after 7 days. All these suggested a rapid healing of corneal injuries in rabbit models.

Said *et al*. (2009) stated that cells originating from the corneal stroma can migrate and populate the amniotic stroma in significant quantities. These observations suggested that the amniotic stroma offers a supportive structure and environment for the repopulation of stromal cells. The stromal cells represented the myofibroblastic and fibroblastic differentiation of keratocytes.

Lu *et al*. (2010) noticed that corneal cells express various growth factors and cytokines during wound healing exerting specific effects on corneal epithelial cells. These included epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor- α and β (TGF-α, and TGF-β), acidic and basic fibroblast growth factors (FGF-1 and FGF-2), insulin-like growth factor-1 (IGF-1),

keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), Interleukins-1,6, and 10 (IL-1, IL-6, and IL-10) and tumor necrosis factor-α (TNF-α). Authors reported that activation of many of these factors occurred during the wound healing process.

Corneal epithelial wounds healed through coordinated actions such as cell migration, proliferation, adhesion, and differentiation, resulting in the stratification of cell layers. This process relied on interactions mediated by growth factors, cytokines, and signals from the extracellular matrix at the wound site. These interactions aided in regaining epithelial integrity and normal function (Ljubimov and Saghizadeh, 2015).

2.9. CLINICAL SIGNS OF CORNEAL INJURY

Gum *et al*. (1999) noticed that disruptions in the rabbit corneal epithelium caused symptoms like tearing (epiphora), involuntary spasms of the eyelid muscle (blepharospasm), redness of the conjunctiva (conjunctival hyperemia), and inflammation of the anterior part of the eye (reflex anterior uveitis). The authors also reported that this uveitis would be manifested as pupillary constriction and the presence of proteins in the fluid of the eye (aqueous flare).

Andrew (2002) reported epiphora (excess tearing) as a clinical indication of corneal disease. Corneal diseases can also result in corneal opacity due to factors like edema and scarring, as well as the development of new blood vessels (neovascularisation).

Mandell and Holt (2005) reported the clinical indicators of corneal trauma as an involuntary spasm of the eyelid muscles, redness of the conjunctiva, ocular discharge from the affected eyes, corneal oedema, and constriction of the pupil. Authors opined that infected ulcers typically presented with a yellow-white infiltration into the corneal tissue, and deep corneal ulcers would have evident corneal neovascularization.

2.9.1. Presence and Nature of Ocular Discharge

Okud and Campbell (1974) noticed that purulent discharge with redness of the conjunctiva indicated conjunctivitis and infections of the nasolacrimal duct. The authors opined that the diagnosis can be solely confirmed by culture specimens from the conjunctival sac in which *Pasteurella* species were rabbits' most common bacterial pathogens.

Williams (2007) stated that ophthalmic emergencies rabbits are characterised by watery discharge from the eyes, involuntary spasms of the eyelid muscles, and swelling of the eyes and eyelids. Ocular discharge resulted in matting of the lateral as well as medial canthus of the eye. The authors recommended collecting corneal swabs for culture and sensitivity since rabbits are prone to bacterial diseases caused by *Pasteurella multocida, E. Coli,* and various staphylococcal species.

2.9.2. Nature and Number of Blinks Per Minute

Evinger *et al*. (1984) stated that photographic evidence of blinking in rabbits and guinea pigs revealed that the upper eyelid moved downward and outward. The authors reported that in blinking, orbicularis oculi played an active role and levator palpebrae played a passive role primarily.

According to Peiffer *et al*. (1994), the typical blinking rate in rabbits is low, usually averaging around 10 to 12 blinks per hour.

2.9.3. Conjunctival Changes

Danjo *et al*. (1987) stated that conjunctival epithelium underwent cell division in response to injury and contributed to the healing process in acute corneal epithelial wounds. The authors proved these through the changes in the rate of cell division in the epithelium and variation in the content of goblet cells.

Shin and Lee (2006) reported that LASIK surgeries in rabbit models using suction rings lead to inflammatory response and conjunctival apoptosis. The authors

opined that whenever there is tissue damage in the cornea or associated structures it could contribute to the occurrence of cell death in the conjunctiva.

2.10. OPHTHALMOSCOPIC EXAMINATION

According to Kern (1997), assessing the eyes of rabbits and rodents could pose challenges due to their small eye size, challenges in restraint, and the need for larger conventional diagnostic tools.

The smallest aperture on the direct ophthalmoscope is ideal for inspecting the anterior chamber to detect aqueous flare. For a thorough examination of the lens and posterior segment, dilation of the pupil can be achieved by using 1% Tropicamide or a mixture of 1% Atropine and 10% Phenylephrine. The animal should be elevated to evaluate the posterior segment, and the examiner should look upward to visualise the optic nerve (Andrew, 2002).

According to Ollivier (2003), corneal examinations using ophthalmoscopy were preferred to evaluate loss of transparency, vascularisation, pigment deposition, abnormal growths, lacerations, foreign bodies, contour change, and ulcerations.

Mitchell (2011) suggested the procedure for direct ophthalmoscopy as, after adjusting the light intensity to a minimum using a rheostat to avoid the patient's discomfort, the examination could be done in such a way that the right eye to examine the patient's right eye and vice versa. Begin by obtaining the tapetal reflection from a short distance to align the image. Start by identifying and examining the optic nerve head, followed by examining tapetal and non-tapetal quadrants, vascular patterns, corneal clarity, etc.

2.10.1. Corneal Clarity

Corneal stroma plays a crucial role in the development of corneal haze following injury. After a corneal injury, corneal oedema resulted in a corneal haze. Haze arising after the initial phases was due to disorientation of the collagen fibrils and accumulation of various macromolecules, including proteins, glycosaminoglycans, and lipids (Moller- Pederson, 2004).

Samuelson (2013) reported that corneal transparency is maintained by the absence of blood vessels, pigment, and myelin in the corneal structure, as well as the organized arrangement of collagen fibrils in a slightly dehydrated state. The author opined that conditions such as diseases, injuries, or surgical interventions can disrupt this transparency.

Sanches *et al*. (2016) developed Royal Veterinary College Corneal Clarity Scores (RVC-CCS), producing reliable reproducibility among observers. The authors found this suitable for evaluating the efficacy of medical or surgical treatments impacting central cornea in small animals. Gradings were ranged from G0 to G4. The scoring system proved effective for assessing lesions in the central cornea but was less optimal for lesions in the peripheral cornea- as the iris obstructed the reflection of the fundus during the evaluation of the peripheral cornea.

2.10.2. Corneal Oedema and Vascularisation

Philipp *et al*. (2000) observed that after corneal injury, corneal epithelial cells, endothelium, stromal keratocytes, immune cells like T-cells, and macrophages released pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF). The authors reported that these vascular endothelial cells secreted enzymes that broke down the vascular basement membrane and the surrounding extracellular matrix of the cornea, allowing them to migrate into the corneal stroma. As they move towards the inflammation site, attracted by chemotaxis, these cells proliferate and form new blood vessels with associated branches.

Tsai *et al.* (2000) stated that corneal neovascularisation was managed surgically by limbal stem cell transplantation, amniotic membrane transplantation, laser therapy, and photodynamic therapy. Similarly, drug treatments included steroids, non-steroidal anti-inflammatory drugs, prolactin, angiostatin, and cyclosporin A.

Chang *et al*. (2001) noted that several angiogenic agents were involved in neovascularisation, and these agents included various growth factors and cytokines, such as b-FGF, VEGF, IL-8, and selectin E. The authors also reported about angiostatic molecules, such as thrombospondin, angiostatin, and platelet factor 4, which inhibit vascularisation.

Burri *et al*. (2004) reported that angiogenesis is the sprouting of new vessel segments and follows specific patterns. The authors opined that the basement membrane will be degraded, endothelial cell proliferation, solid sprouts of endothelial cells connecting a neighboring vessel, and restructuring of the sprout into a lumen lined by endothelial cells and formed as a vascular network.

Corneal neovascularization was regulated by local pro-angiogenic and anti-angiogenic factors, and the equilibrium between these factors typically maintained corneal avascularity. Corneal neovascularization was triggered by increased expression of angiogenic cytokines in response to inflammation or hypoxic conditions (Azar, 2006).

Costagliola *et al*. (2013) reported that the metabolic pump within the endothelium was the primary factor affecting corneal deturgescence. The pump facilitated the movement of fluid out of the corneal stroma into the aqueous humor through active transport mechanisms. The process relied on oxygen and ATP for energy; lack of any of these could lead to corneal oedema.

Brooks *et al.* (2017) mentioned neovascularisation of the cornea as a normal phenomenon and a reparative response to injury of the corneal surface.

2.11. NEURO-OPHTHALMIC TESTS

2.11.1. Pupillary Light Reflex

Clarke *et al*. (2003) defined pupillary light reflex as the narrowing of the pupil triggered by an increase in light on the retina. Direct PLR occured when the pupil in the same eye constricted as that stimulated with light. Conversely,

consensual PLR involves the pupil in the opposite eye, constricting light stimulation.

Maggs (2008) explained PLR as not an indicator of vision but merely a subcortical response. Whenever there was physical obstruction of iris, iris atrophy, and excitement due to adrenaline rush in animals there were reduced response or no response at all.

Adhikari *et al*. (2015) defined Pupillary Light Reflex (PLR) as a physiological measure of the nervous system's normal or abnormal functioning. The symmetry of the PLR in response to stimulation of either eye, owing to the decussation of pupillary fibers, presented an opportunity to compare the pupillomotor drive in both eyes.

Warnefors *et al*. (2019) observed that the direct pupillary light reflex was slower and less pronounced in rabbits than in dogs. The consensual pupillary light reflex observed was feeble and had limited diagnostic significance in rabbits. This could be attributed to the fact that only 10% of the optic nerve fibres decussate in rabbits.

2.11.2. Dazzle Reflex

The Dazzle or photic blink reflex can be activated by shining a bright light onto the back of the eye and noting the resulting blink response. The reflex operates at a subcortical level, with the highest reflex centre believed to be the rostral colliculi. The facial nerve serves as an efferent pathway for this reflex. Gelatt (1997).

According to Martin (2001), for a dazzle reflex, there should be an intact retina, optic nerve, optic tract, optic chiasma, supraoptic nuclei, and rostral colliculi. It was explained as the bilateral narrowing of the palpebral fissure when a very bright light stimulates the retina.

Mandell and Holt (2005) reported that menace and dazzle reflexes were valuable for evaluating the retina and optic nerve. The authors also noticed that this is challenging in animals having pain due to partial closure of eyes.

Maggs (2008) opined that the dazzle reflex was triggered by sudden exposure to intense light, prompting a swift blinking reaction and sometimes reflexive movement to turn the head away. Reflex operated on a subcortical level and served as a tool to differentiate cortical blindness from blindness caused by damage elsewhere on the visual pathway, whereas the dazzle reflex would be absent if the damage is complete.

2.12. SPECIAL DIAGNOSTIC PROCEDURES

2.12.1. Schirmer Tear Test

Ochipinti *et al*. (1988) stated Schirmer teat test as a diagnostic aid to rule out keratoconjunctivitis sicca. General clinical use of the test involved placing a standard notched filter paper into the lower conjunctival fornix of the eye and reading the wetness of the paper after one minute.

Abrams *et al*. (1990) reported tear production measurements in rabbits. The mean standard deviation (SD) STT value in normal rabbits was found to be 5.30± 2.96 mm/min (with a range of 0-15 mm/min), and significant differences were noticed among breeds.

Birick *et al*. (2005) observed that the Schirmer tear test is suitable for rabbits and guinea pigs, and the test strip is too large for rats and mice. Significant breed-wise differences were also evident, with Netherland Dwarf rabbits having an unusually high value of 12 ± 2.5 mm/min compared to an average of 5.3± 2.9 mm/min with other breeds.

Petznick *et al*. (2011) defined STT I, which was performed without the use of topical anesthesia, provided a measure of both basal and reflex tearing, whereas STT II, which was performed after topical anesthesia with a drop of tetracaine, demonstrated basal tearing alone. Basal tears were those produced in the absence

of stimulation and were lacrimal secretions that maintained the ocular surface. Reflex tears were produced after stimulation, usually as a result of irritation to the surface, such as a foreign body or an inflammatory response. The tear strip itself likely acted as a source of irritation to increase tear production in STT 1.

Lima *et al*. (2015) reported that qualitative tests used to assess tear film include the Schirmer tear test (STT), Phenol Red tear test (PRTT), and Endodontic absorbent paper point tear test (EAPTT). In New Zealand white rabbits, normal tear test results noticed were approximately 4.8 ± 2.9 to 5.3 ± 2.9 mm by STT, 20.8 ± 3.7 mm by PRTT, and 13.8 ± 1.5 mm/min by EAPTT. Comparatively, tear production values obtained from English Angora and Dutch rabbits were similar to those of New Zealand white rabbits. Results of PRTT and EAPTT indicated higher tear production in Angora and Dutch rabbits.

Maggs *et al*. (2017) noted that lagomorphs Schirmer tear test results were lower than felines. Despite rabbits blinking only in every five to six minutes, their tear film remains relatively stable, and they rarely suffer from exposure keratitis due to evaporation. Tear film stability in rabbits was achieved by the presence of the lacrimal gland, accessory lacrimal gland, superficial gland of the third eyelid, and the harderian gland. Additionally, the lower Schirmer tear test result in rabbits compared to felines can be attributed to the retention of the tear film due to the absence of superior lacrimal puncta.

Variations in STT I results may also result from factors beyond tear production, such as the emotional state of the animal during the examination, differences in Schirmer strips used, and variations in ambient humidity levels within the testing environment (Yoon *et al*., 2020)

Corsi *et al*. (2022) opined that evaluating the quality and quantity of tears in veterinary medicine is crucial for diagnosing and managing ocular conditions. The most common methods used were the Schirmer Tear Test (STT), tear film osmolarity measurement, interferometry, tear meniscus height measurement, and

Meibography. Each method provided valuable information about tear film quality, quantity, and ocular surface health.

2.12.2. Fluorescein Dye Test

Bedford (1982) noted that after taking up the fluorescein stain corneal stroma appears brilliant green under cobalt blue light during the course of a corneal epithelial defect.

Foulks and Pavan-Langston (1985) opined that fluorescein has been utilized in clinical practice to identify corneal epithelial abrasions associated with different types of punctate keratitis or epithelial defects.

Feenstra and Tseng (1992) used fluorescein staining while using amniotic membrane to treat corneal surface irregularities. The authors noticed that fluorescein dye has superior penetration capabilities compared to lissamine green through the corneal stroma.

Corneal stroma, which is hydrophilic, can only retain water-soluble fluorescein dye (Wilkie and Whittakkar, 1997).

Korb (2000) stated that sodium fluorescein and rose bengal dyes have served as the primary dyes for assessing ocular surface disturbances. Fluorescein is recognized as the primary dye for corneal staining, and rose bengal is the primary dye for conjunctival staining. These dyes are extensively used in clinical settings to diagnose conditions related to tear film and dry eye syndrome.

According to Miller (2001), a Fluorescein dye test is performed by instilling a drop of normal saline over an impregnated strip, and diluted dye is allowed to contact the superior bulbar conjunctiva. After allowing the animal to blink the excess stain is washed off for examination.

Costa *et al*. (2019) studied the re-epithelialization of the cornea after grafting corneal ulcers with an amniotic membrane. The author confirmed corneal re-epithelialisation through a negative fluorescein dye test

2.13. CULTURE AND ANTIBIOTIC SENSITIVITY

Every corneal ulcer would undergo scraping and direct inoculation onto culture plates. These plates would then be maintained under suitable conditions in a microbiological laboratory without further processing. If the predetermined criteria for empiric therapy were satisfied, treatment could be initiated accordingly. If the ulcer shows no improvement the interpretation of culture and sensitivity is important (Macleod *et al*., 1996).

Beegum *et al*. (2006), in a study of bacterial isolates from corneal specimens, reported that from the positive cell cultures, majority were attributed to Gram-positive bacteria, with coagulase-negative Staphylococci being the most prominent, followed by Streptococcus pneumoniae, among the Gram-negative bacteria isolated Pseudomonas accounts for positive cultures. All the isolates demonstrated sensitivity to ofloxacin and other ocular antibiotics.

Tolar *et al*. (2006) noticed that corneal culture swabs of dogs showed increased resistance to bacitracin, chloramphenicol, and cephalothin because of the greater prevalence of isolates of *Pseudomonas aeruginosa.* Resistance to fluoroquinolone antimicrobials remained minimal, suggesting ciprofloxacin as the effective treatment for bacterial keratitis in dogs. Tobramycin was preferred over gentamicin due to its lower toxicity to epithelial cells in vitro. On the other hand, resistance of gram-positive bacterial isolates to fluoroquinolones was on the rise.

2.14. HISTOLOGICAL OBSERVATIONS DURING CORNEAL HEALING

Nishida *et al*. (1990) noted that activated keratocytes possess phagocytic properties against foreign materials and play a role in stromal collagen breakdown by producing collagen-degrading enzymes. This mechanism explained the resorption of the amniotic membrane without concurrent stromal vascularisation.

Gris *et al*. (2002) opined that the histopathological examination of the cornea after amniotic membrane grafting revealed initial re-epithelialisation with

5-7 layer thickness of epithelium while partial restoration of stromal thickness. The authors also noted a thin band of eosinophilic infiltrate over the central cornea corresponding to the amniotic membrane graft. Regarding stromal healing, the authors opined that the amniotic membrane initially filled the defect and later got substituted by fibroblasts and collagen

Wang *et al*. (2001) assessed the corneal healing by amniotic membrane in rabbits after excimer photoablation. All corneas were collected using a 9 mm trephine and halved to traverse the ablated centre. Three corneas were preserved in glutaraldehyde 2.5%, subjected to Hematoxyllin-Eosin as well as Masson's trichrome staining, and scrutinised via high-power magnification under light microscopy to evaluate epithelial hyperplasia and stromal fibroblast cellularity.

Dua *et al*. (2004) stated that when used as a patch, the amniotic membrane typically detached prematurely, sometimes before the desired time, or was eventually expelled. Histological examination revealed fibrillar remnants of the amniotic membrane. The membrane's disintegration often manifested as noticeable large gaps beneath the overlying epithelium.

Tosi *et al*. (2005) histologically evaluated corneas after amniotic membrane transplantation and reported that re-epithelialisation, arrangement of newly formed epithelium, presence or absence of amniotic membrane, arrangement of stromal collagen and fibroblasts, presence or absence of PMN cells, neovascualrisation, oedema, etc. are evaluated during the healing process of cornea.

Said *et al*. (2009) reported that when amniotic membranes were transplanted over corneal defects, corneal stroma-derived cells (CSDC) migrate from corneal stroma and repopulate in the amniotic stroma. The authors also reported that during the initial phases of healing, corneal stroma-derived cells extended to the superficial amniotic stroma, while in the later stages, they get repopulated to the deeper layers of the amniotic stroma, providing better acceptance of the graft.

Capistrano da Silva *et al*. (2021) studied the effect of Bovine Amniotic Membrane Homogenate in rabbit ex-vivo corneas by collecting them from the

culture plate on the 7th day, immersed in 10% buffered formalin for 48 hours, moved to an ethanol solution before being embedded in paraffin. Spanning from limbus to corneas were trimmed and then embedded in paraffin. Corneas were horizontally sectioned in a 5 µm thickness and stained with Hematoxyllin and Eosin. On histological analysis, no keratocyte deterioration was evident, and on the healed region, a consistent epithelial layer with basal and superficial cells was observed.

3. MATERIALS AND METHODS

The present study was conducted on 18 healthy adult rabbits procured from the Committee for Control and Supervision of Experimentation on Animals (CCSEA) approved breeding station - Small Animal Breeding Station- Mannuthy. The study was conducted at the Department of Veterinary Surgery and Radiology, Teaching Veterinary Clinical Complex, College of Veterinary and Animal Sciences, Pookode.

Central corneal defect of six-millimetre diameter was induced in the right eye of all the rabbits of which nine of them served as the test group while the rest served as the control group. The test group received decellularised bovine amniotic membrane (BAM) derived extracellular matrix (ECM) scaffold as a graft on the cornea along with topical antibiotic eye drops and oral anti-inflammatory, while the control group received topical antibiotic eye drops and oral anti-inflammatory alone. Healing of the cornea following grafting was evaluated on days 7, 14, and, 28 post-surgery.

3.1. HARVESTING, PREPARATION AND PRESERVATION OF BOVINE AMNIOTIC MEMBRANE DERIVED EXTRACELLULAR MATRIX SCAFFOLD (PLATE 1a, 1b and 2)

Bovine amniotic membranes (BAM) were obtained aseptically from cows that underwent caesarean section. The amnio-chorion thus collected was cleaned off blood clots by washing with normal saline solution. The amnion was then peeled from the chorion. The obtained samples were then washed in phosphate-buffered saline (PBS) containing mixture of Penicillin[1] (50µg/mL), Streptomycin[3] (50µg/mL) and Amphotericin B[2] (2.5µg/mL). Thereafter, the samples collected were washed in cold PBS. The bovine amniotic membranes (BAMs) were then subjected to a freezing cycle in liquid nitrogen (-196^{0}C) for 22 hours followed by unfreezing in a water bath at 37^{0}C for two hours. The membrane was then immersed

in Tween 80 for four hours. BAMs were rinsed with distilled water to remove the remnants of the reagent. After each decellularization step, this rinsing procedure was repeated to remove remnants of the reagents. BAMs were then immersed in 0.1 M NaOH for one hour, later in 0.1 M ascorbic acid for 12 hours. The membranes thus obtained was dipped in 70 % Ethanol for one hour to remove residual nucleic acids and phospholipids and finally washed with PBS for two hours. During the entire procedure, membranes were mechanically stirred in an orbital shaker to ensure a homogenous wash. The decellularised membrane thus obtained was kept at -20^0 C in sterile containers till further use (Ballesteros *et al.,* 2020). The final sample was subjected to histological studies to rule out cellularity. The decellularised amniotic membrane revealed lack of epithelium and absence of cellularity stroma.

3.2. SURGICAL PROCEDURE (PLATE 3)

3.2.1. Preoperative Preparation

The eyelashes were clipped and area around the eye was cleansed with 5% povidone-iodine solution. The eye surface and conjunctival fornix were prepared aseptic with diluted (0.5 percent) povidone-iodine solution followed by irrigation with sterile isotonic saline.

3.2.2. Anaesthesia

Animals were anesthetized with intramuscular administration of Xylazine[4] @ 5 mg/kg body weight and Ketamine[5] @ 50 mg/kg body weight.

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[1]CRISPEN-5®, 500000 IU/vial, Alembic Pharmaceuticals Ltd, Vadodara, Gujarat

[2]AMFOCARE® -5 mg/ml - Bangalore Pharmaceutical and Research Laboratory PVT Ltd, Bangalore

[3]AMBISTRYN S® 1g, Abbot Healthcare Pvt. Ltd, Mumbai, Maharashtra

[4]XYLAXIN® 20mg/ml, Indian Immunologicals Ltd, Telangana

[5]ZOKENT® 50mg/ml, Miracalus Pharma Pvt Ltd, Mumbai, Maharashtra

3.2.3. Preparation of Central Corneal Defect

All animals (test and control group) were positioned on left lateral recumbency with the eye to be tested positioned above. Eyeball was fixed in a central position with stay sutures. Circular central corneal wounds, of 6 mm diameter – involving epithelium and anterior stroma, were created using a corneal trephine. Histological examination of removed cornea after Haematoxylin and Eosin staining revealed an anterior stratified squamous epithelium of non-keratinising type of 5-8 cell layers thick. The layer presented tall columnar basal cells, two to three layers of polyhedral cells covered by two to three layers of flat squamous cells. The basal cell layer rested on a thick basement membrane. The deep part of the defect revealed a part of the substantia propria forming the corneal stroma consisting of several lamellae of collagen layers and enclosing fibrocytes or keratocytes between the fibres.

3.2.4. Grafting of Cornea with Decellularised BAM-Derived ECM Scaffold

The preserved decellularised BAM-derived ECM scaffold was rinsed in sterile saline and spread over nitrocellulose filter paper. A circular graft of 6 mm diameter was harvested from this decellularized membrane using the trephine. The circular BAM scaffold was layered over the corneal defect and secured to the margins using 10-0 nylon in the test group. A third eyelid flap was done and maintained for three days, to protect the sutured graft. Both test and control group were treated with topical antibiotics eye drops and oral anti-inflammatory for one week.

3.2.5. Post-Operative Care

An elizabethan collar was provided in all the cases to prevent self-mutilation. Tobramycin eye drops and meloxicam at 0.1 mg/kg body weight were administered for five and three days respectively in both groups. Three animals from each group were sacrificed after ophthalmic examination on 7, 14 and, 28 days post-surgery to collect cornea for histopathological studies. The maintenance as well as sacrificing of rabbits will be done as per CCSEA guidelines.

3.3. MAIN ITEMS OF OBSERVATION

All animals were subjected to detailed ophthalmic examination on 7, 14, and, 28 days post-surgery.

3.3.1. Presence and Nature of Ocular Discharge

The eyes of the animal were observed for lacrimation and the nature of ocular discharge. The nature of ocular discharge was recorded as serous, mucoid, mucopurulent, or purulent. The quantity of ocular discharge was noted as mild, moderate, and profuse.

3.3.2. Results of Visual Function Tests

Visual function was assessed for the tested eye on all days of observation using pupillary light reflex (PLR) and Dazzle Reflex.

For PLR, a light source was projected into the tested eye. Resultant pupillary constriction in the same eye was observed and noted as sluggish, present, or absent.

For Dazzle Reflex, a high-intensity light was flashed into the tested eye in a dark room, eliciting a partial or complete narrowing of palpebral fissure. The result was observed and noted as positive or negative.

3.3.3. Nature and Number of Blinks per Minute

The blinking of third eyelid was observed and noted as present or absent in each animal upon rest. The number of blinks per minute was also recorded.

3.3.4. Conjunctival Changes

All the animals were observed for conjunctival changes and noted as generalized congestion, injection of vessels, or normal.

3.3.5. Results of Ophthalmoscopic Examination

Ophthalmoscopic examination was done using hand-held ophthalmoscope (Welch Allyn, 11770 Inc.) and the findings were noted and recorded. Corneal changes after grafting, corneal clarity, corneal oedema, vascularisation of cornea

and extent of pigmentation if any were examined and observed using the ophthalmoscope.

3.3.5.1. Corneal Clarity

The clarity of the cornea was assessed and assigned scores of 4+ to 1+. Clarity was graded as clear (4+) if retro illumination was clearly visible in the central corneal defect, hazy (3+) if the pupil was visible, but retro illumination was not clearly visible in the central corneal defect, moderate opacity (2+) if the pupil was not visible in the central corneal defect and retro illumination was slightly visible, and complete opacity (1+) if retro illumination was obscured.

3.3.5.2. Corneal Oedema and Vascularisation

Corneal oedema was assessed and subjectively graded as 0 (no signs of oedema), 1 (mild oedema), 2 (marked oedema) and 3 (severe oedema).

Vascularisation of the cornea on the tested eyes were examined and subjectively graded based on degree of vascularisation, graded as 0 (no visible vessels), 1 (mild superficial vascularisation), 2 (profuse superficial vascularisation) or 3 (extensive vascularisation with vessels originating from all quadrants of cornea).

3.4. SPECIAL DIAGNOSTIC TESTS

3.4.1. Results of Schirmer Tear Test

Schirmer tear tests - (STT I and STT II) were done using sterile Schirmer tear test strip (OpStrip, Ophtechnics Unlimited) with printed millimetre scale on it, and the observations were noted. For STT I (reflex tear production), the strip was placed into the ventral conjunctival cul-de-sac for one minute and the distance of wetness was measured immediately and noted, and compared against normal range.

For STT II (basal tear production) cornea and conjunctival cul-de-sac was desensitised using proparacaine and the excess proparacaine was blotted away with

a cotton wick after a few minutes. The strip was then placed in the ventral cul-de-sac as that for STT I and the distance of wetness was measured to note the basal tear production.

3.4.2. Results of Fluorescein Dye Test

Fluorescein dye test was done using sterile dye-impregnated strips (Fluoro Touch, Madhu Instruments Pvt. Ltd). The fluorescein dye-impregnated tip was held against the bulbar conjunctiva and two drops of sterile saline was instilled on it for the dye to elute and fall on the eye. The animal was allowed to blink to distribute the fluorescein dye across the ocular surface. The excess stain was flushed out with sterile normal saline. The eye was then examined under cobalt blue light and the findings were noted and recorded.

3.4.3. Results of Culture and Antibiotic Sensitivity of Corneal Swab

The corneal swabs for antibiotic sensitivity were collected using sterile cotton swabs on days 7, 14, and 28 post-operatively before instilling any medications. The eyelids were gently retracted and the sides of moistened sterile swabs were rolled over the grafted cornea without touching the eyelid margin or eyelashes. These samples were inoculated in brain heart infusion agar for bacterial culture on the day of sample collection, incubated anaerobically at 37^0C for 24 hours and observed for bacterial growth. From there it was inoculated into Muller Hinton agar. Antibiotic discs used were Enrofloxacin, Gentamicin, Moxifloxacin, Tobramycin, Ciprofloxacin, Ofloxacin, and Amikacin. The zone of inhibition diameter was measured for each antibiotic. The results were compared with an interpretative chart furnished by the manufacturer and the sensitivity of the organism was recorded as (S) sensitive, (I) intermediate, and (R) resistant, (CLSI, 2018)

3.5. HISTOLOGIC OBSERVATION DURING HEALING PROCESS

Following ophthalmoscopic examination, three animals from each group were sacrificed humanely on days 7, 14, and 28 post operatively. The tested corneas were harvested for histological evaluation to study the healing. The corneas were washed thoroughly in normal saline and fixed in 10 % buffered formalin for 48 hours and processed routinely for Haematoxylin and Eosin staining as per the standard procedure (Fischer *et al*., 2008). The stained sections were examined for degree of corneal oedema, reepithelialisation, infiltration of inflammatory cells, neovascularisation, and fibroblast proliferation.

3.6. STATISTICAL ANALYSIS

The values obtained were analysed. Comparison between group 1 and group 2 animals for each day of observation was done by using independent t-test using SPSS version 24.0.

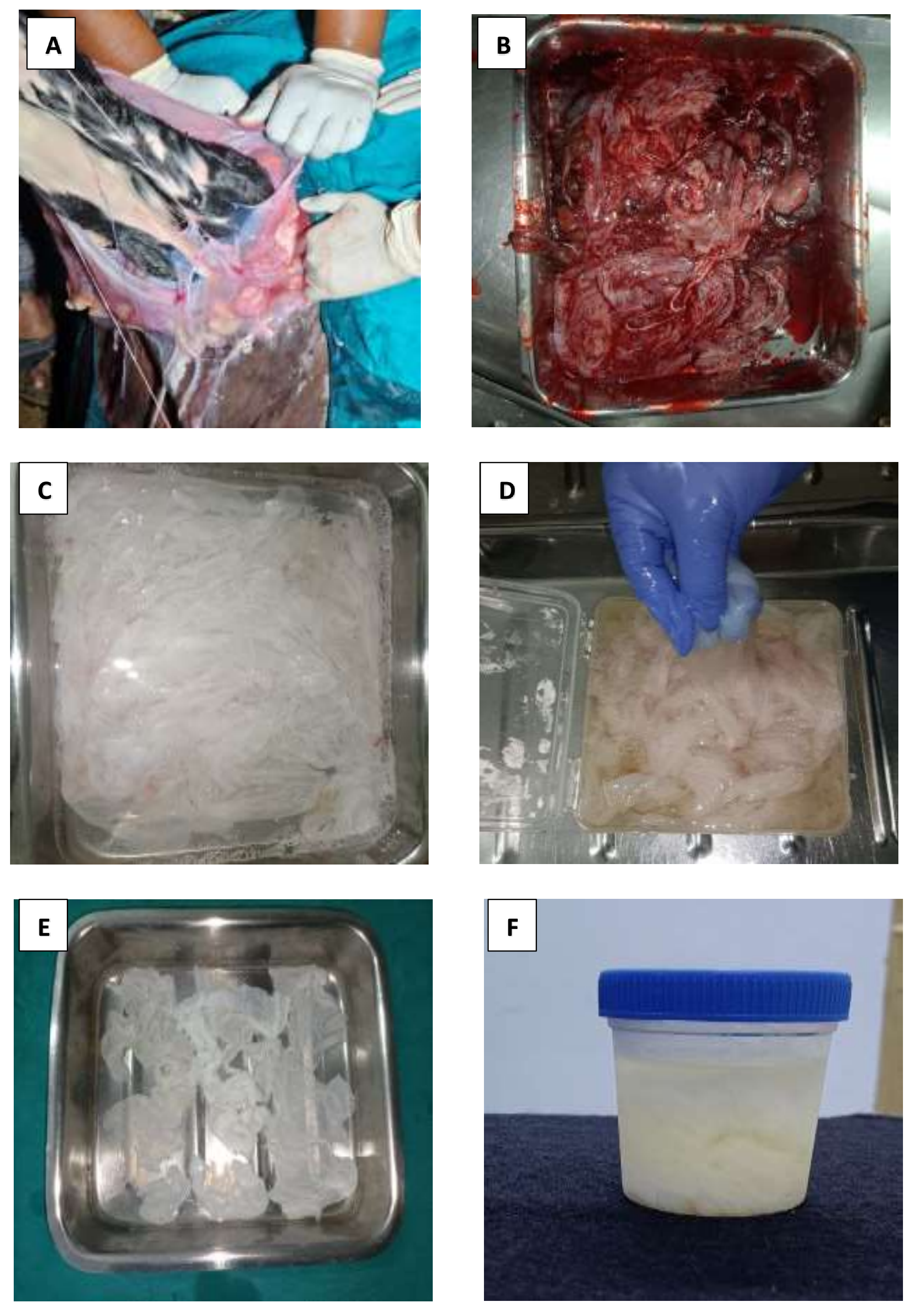


Plate 1a. Collection of bovine amniotic membrane. (A) Collection during Caesarean section, (B) Amniotic membrane in sterile tray, (C) Rinsing, (D) Peeling chorion from amnion, (E) Amnion in sterile tray, (F) Amniotic membrane stored in PBS

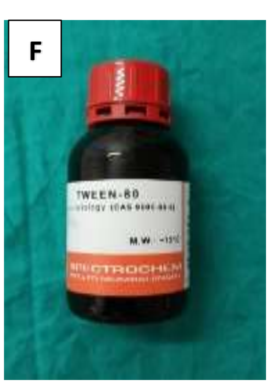


Plate 1b. Materials for BAM collection and decellularisation. (A) Cryocan, (B) Water bath, (C) Orbital shaker, (D) Streptomycin, Amphotericin, and Penicillin, (E) Phosphate buffered saline, (F) Tween 80, (G) Sodium hydroxide, (H) Ascorbic acid, (I) Ethanol

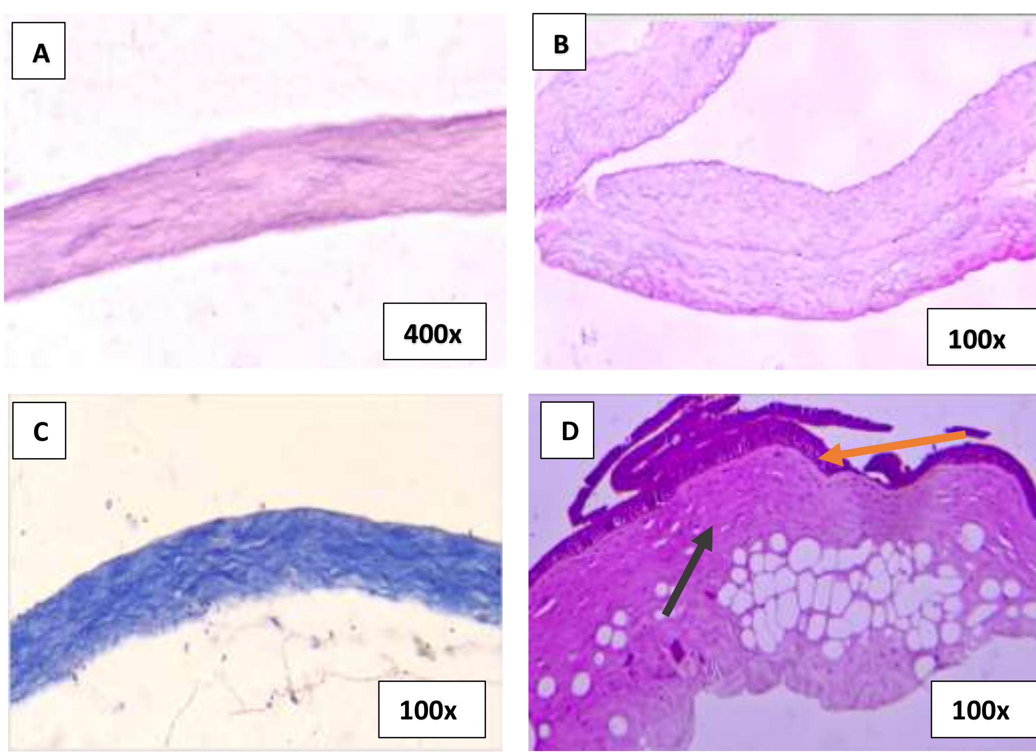


Plate 2. Histology of decellularised BAM (Fig A, B, and C) and corneal defect (Fig D). (A) and (B) Decellularised BAM showing collagenous stroma lacking surface epithelium, (C) Gomori's one step trichrome staining showing collagenous stroma of BAM, (D) Histology of removed corneal portion – orange arrow indicates epithelium, black arrow indicates stroma

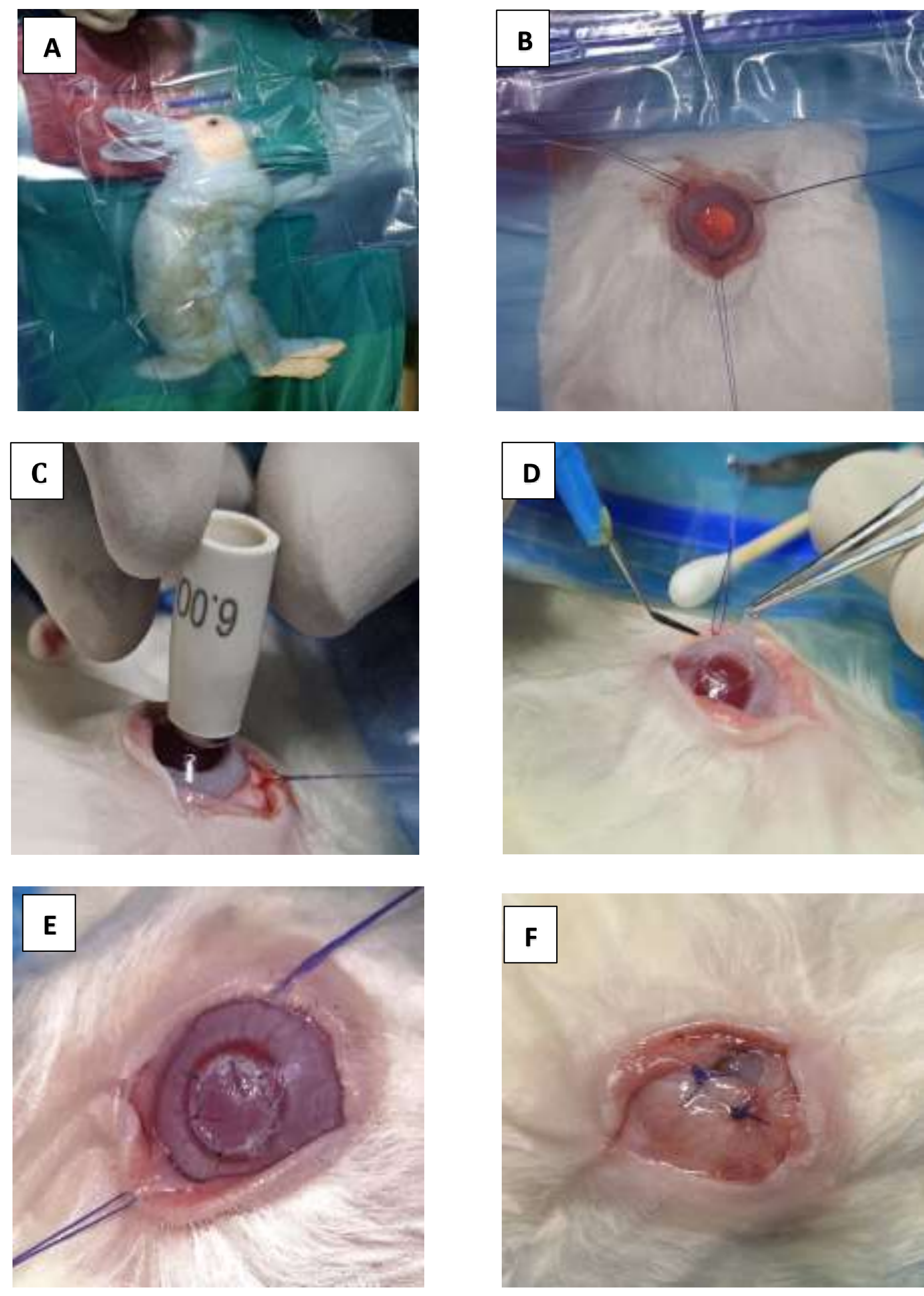


Plate 3. Surgical procedure. (A) Positioning of animal, (B) Fixation of eyeball, (C)Creation of central corneal defect using six millimetre corneal trephine, (D)Removing corneal epithelium and anterior stroma for creating the defect, (E)Amniotic membrane as inlay graft, (F) Third eyelid flap

2. REVIEW OF LITERATURE

2.1. ANATOMY OF RABBIT EYE AND CORNEA

According to Davis (1929), the rabbit cornea is unusually prominent and wide. Its radius of curvature measured about 7.3 mm, with its shape roughly elliptical and having a longer horizontal axis. The horizontal diameter averaged about 15.6 mm., and the vertical about 13.8 mm. The thickness of the cornea was fairly uniform, being slightly greater at the limbus. Thickness averaged 0.37 mm at the centre and 0.45 mm near the limbus.

Maurice (1995) stated that the orbital glands, including the large Harderian gland, produced tears, which explains the exceptionally slow blink rate - one or two blinks per minute in rabbits. According to the authors, the exceptionally slow blink rate has deleterious consequences on corneal epithelial healing and drug delivery.

Beurman and Pedroza (1996) reported that Descemet's membrane is the thick basement membrane of the corneal endothelium and is very rich in collagen. Authors also reported that corneal endothelium comprised of an active metabolic pump which helps in maintaining corneal clarity.

Ojeda *et al*. (2001), through scanning electron microscopic (SEM) studies, documented that corneal epithelium appeared smooth, on which the cell boundaries could not be resolved. The epithelial surface was randomly infiltrated by numerous nerve fibers. The authors also reported that the rabbit epithelial basement membrane (EBM) appeared as a flat network of straight fibers with no defined order. also, the stroma comprised stromal lamellae, keratocytes, and stromal neural plexus.

Andrew (2002) stated that 30% of the eyball in rabbits comprised of cornea, with dimensions of approximately 15 mm horizontally and 14 mm vertically. The authors also reported that the layers of the cornea from outer to inner, comprised of the epithelium, which is a keratinized layer 30-40 micrometers thick, then followed by the stroma which is the thickest layer, made up of collagen bundles arranged

parallelly, followed by Descemet's membrane which is 7-8 micrometers thick and continuously getting thickened with age and reaching 15 micrometers thick in aged rabbits, and finally the inner endothelium, which is single cell layer containing crucial ATP-ase pump that played a major role in maintaining corneal transparency.

Klenker *et al*. (2007) opined that corneal epithelium is protected by an anterior tear film, which prevents damage and entry of pathogens and contributes to growth factors like epidermal growth factor (EGF), which promotes epithelial regeneration.

According to Samuelson (2013), the third eyelid lacked muscles but was passively drawn more than two-thirds of the way across the eyeball by the pull of the powerful retractor oculi muscles. The lack of an orbital fat pad rendered the rest of the orbit filled only by the retrobulbar muscles, the orbital glands, and the globe itself. Tear drainage is through a single slit-like opening 3–4 mm from the eyelid margin. There was no defined lacrimal sac as in the dog, cat, and human, but rather an elongated dilatation of the nasolacrimal duct.

Bukowiecki *et al*. (2017) classified layers of cornea as anterior epithelial layer, epithelial basement membrane, stromal layer, Descemet's membrane, and an endothelial cell layer. The authors reported that this stromal layer comprised of tightly packed collagen fibers and sparsely spread keratocytes. The Descemets membrane constitutes the basement membrane for the endothelium. The endothelial cell layer served as the innermost portion of the cornea and was in direct contact with aqueous humor.

2.2. PHYSIOLOGY OF RABBIT CORNEA

Hughes (1972) stated that rabbits, as a prey species, possessed a visual system that concentrated on the horizon. This allowed them to gather visual input for almost 360 degrees of circumference providing an almost complete visual field.

Klyce and Crosson (1985) opined that corneal epithelium helped in corneal hydration, but its fluid-transporting ability was many times less than that of

endothelium. The endothelial pump normally functions at some fixed capacity, but activity becomes questionable when there was an underlying pathology or ageing.

Bukowiecki *et al*. (2017) mentioned that the cornea lacked blood and lymphatic vessels. The cornea receives its oxygen and nutrients through tear film on its outer side, aqueous humor on its inner side, and limbal vasculature on its periphery.

2.3. RABBIT AS AN EXPERIMENTAL MODEL

Rabbits have been used as a model for studying radiation-induced cataracts since the 1950s. Investigators performing radiation studies in rabbits observed that cataracts developed similar in structure to radiation-induced cataracts in humans. (Cogan and Donaldson, 1951).

Gimborne *et al.* (1974) studied prevascular tumor growth and the host's angiogenic response to live tumor cells and tumor-angiogenesis factors-3, 6 in rabbit cornea. Implantation at varying distances from the circumferential limbal vessels produced an anatomic separation of tumor cells from responding host vessels, allowing independent observations of both elements' behavior.

Myles *et al*. (2005) reported that rabbits, considering the size of their eyes, are one of the most widely used species, to investigate novel methods of ocular drug delivery to the posterior segment for treatment of vitreoretinal diseases.

The significant size of the rabbit eye relative to its body size makes them a desirable and cost-effective choice for conducting surgical and drug delivery experiments. The eyes of rabbits shared few similarities with human eyes. The rabbits have thinner cornea compared to humans and a larger lens, causing the iris to bulge forward, creating a more curved anterior chamber (Werner *et al*., 2006).

2.4. INDUCED CORNEAL WOUNDS

Jester *et al*. (1992) employed radial nonpenetrating corneal incisions for studying corneal healing in rabbits and cats. Incisions were made with the help of a diamond knife with its micrometer adjusted to 85 percent of the central corneal

thickness as determined by ultrasonic pachymetry. A three mm central optical zone centered above the pupil was demarcated using an optical zone marker. Radial incisions started at the central optical zone and extended peripherally to 2-3 mm inside the limbus.

Stepp *et al*. (1993), in their review on corneal wound healing studies, reported that the typical wound size in rabbits is 5 or 6 mm while it is 3 mm in rats. The authors also reported that Wounds were created with the help of trephines of different diameters to demarcate the area within which tissue is removed. Sharp trephines penetrated tissues easily. The trephines were dulled using Arkansas stone or similar tools to prevent them from penetrating the stroma.

Wang *et al*. (2001) performed transepithelial ablation using the VISX Star laser under a phototherapeutic keratectomy (PTK) mode using an ablation diameter of 6.0 mm and a depth of 120 mm without edge smoothing for their studies in rabbit corneas. In their study, one eye was used as a test while the other served as a control. The keratectomy wounds were covered with a preserved human amniotic membrane of 9 mm diameter.

Li *et al*. (2016) mechanically debrided the limbus in New Zealand white rabbits using Algar Brush II rotating burr for creating and studying limbal stem cell deficiency. The debridement was done in such a way that the burr tip was applied with light pressure and in a circular motion starting at the 12 o'clock position of the eye and slowly moving around the entire periphery of the cornea.

Capistrano da Silva *et al*. (2021) experimented with bovine amniotic membrane homogenate for healing studies on ex-vivo corneas in rabbits. A 6 mm diameter and 0.125 mm depth circular keratectomy was made using a Hessburg-Barron vacuum corneal trephine and crescent knife in the central cornea. Following creating a corneal wound, corneoscleral rims were aseptically harvested by incising circumferentially approximately 7 mm posterior and parallel to the limbus.

2.5. BOVINE AMNIOTIC MEMBRANE

Schwarze (1972) studied the amniotic membrane in ruminants and reported that the lining of the amniotic cavity in ruminants was clear and lacked significant blood supply while they had extensive connections with the chorion. There were abundant glycogen-rich villi or plaques in the amniotic membrane, which formed around the third month of gestation.

Lee and Tseng (1997) noticed that the amniotic membrane served as a platform for epithelial cell growth and axonal regeneration. The authors also reported that the amniotic membrane supports epithelialisation by aiding the migration of epithelial cells, strengthening their adhesion to the underlying layer, promoting differentiation, and preventing cell death through its anti-apoptotic properties.

Tseng *et al.* (1999) stated that amniotic membrane has anti-inflammatory properties through down-regulation of TGF-beta signaling systems. The authors reported the presence of IL-1 receptor antagonists and IL-10 in the amniotic membrane, potent inflammation inhibitors. The authors studied and reported that the anti-scarring effects of the amniotic membrane were due to the prevention of fibroblast activation into myofibroblast.

Hao *et al*. (2000) reported that fresh and preserved amniotic membranes have anti-angiogenic effects, and these effects were enhanced by chemicals such as thrombospondin-1, endostatin, and inhibitors of metalloproteases.

Koizumi *et al*. (2000) reported the presence of growth factors such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and transforming growth factors β (TGF-β) in the bovine amniotic membrane.

Schlafer *et al*. (2000) stated that the bovine fetal membrane consists of the chorion, the allantois, and the amnion. During development, the amniotic

membrane partially fuses with the chorion, forming the chorioamnion, and partially with the allantois forming the allantochorion. The authors also stated that the amnion typically appears white and smooth apart from chorion or allantois which is red or velvety.

Amniotic membrane or amnion is a highly suitable option for grafting due to its pluripotent cell properties. Serving as the innermost layer of the placenta, it lacked blood vessels and performed various roles such as the transport of water and soluble materials and the production of bioactive factors, including vasoactive peptides, growth factors, and cytokines (Toda *et al*. 2007).

Niknejad *et al*. (2008) observed that the extracellular matrix of the amniotic membrane consisted of collagen I, III, IV, V, VI, and XV, along with laminin, nidogen, fibronectin, and proteoglycans. The authors reported the presence of these collagen in extracellular matrix structure hold promise for amniotic membrane as a biomedical material for tissue engineering.

Park *et al*. (2008) reported that. histologically amniotic membrane comprised of a single layer of cuboidal epithelium. The authors also documented that acellular bovine amniotic membrane proved to be a reliable and efficient dressing material. Bovine amniotic membrane enhanced wound healing capabilities and acted as a barrier against bacterial infection. Massive production and long-term storage were possible and were easy to apply.

Kim *et al*. (2009) reported promising efficacy with bovine freeze-dried amniotic membrane in the healing of corneal epithelial wounds in dogs. The authors also reported that bovine freeze-dried amniotic membrane would be a viable alternative treatment for canine superficial corneal ulceration, surpassing conventional therapies.

Mamede *et al*. (2012) observed histologically that, the amnion is composed of epithelial cells, an acellular basement membrane, and mesenchymal cells. The layer that was adjacent to the amniotic fluid was the inner layer, which was composed of a single homogeneous layer of cuboidal epithelial cells firmly attached

to the basement membrane. The basement membrane was comprised of abundant heparan sulfate, acting as a permeable barrier to macromolecules and was attached to a condensed acellular layer of collagens type I, II, and V.

Litwiniuk and Grzela (2014) opined that corneal ulcers and other ocular disorders, such as perforation, chemical or thermal burns, keratomalacia, etc, could be treated by an amniotic membrane. Authors stated that Amniotic membrane (AM) has been popular because of its biocompatibility, anti-proteolytic, anti-angiogenic, anti-inflammatory, antifibrotic, anti-scarring, antibacterial, and reepithelializing properties.

Min *et al*. (2014) reported better aesthetic results and healing after applying BAM for wound repair on the skin surface. Authors reported that histologically amniotic membrane consists of cuboidal as well as flattened epithelial cells and mesenchymal connective tissue. Epithelial cells migrate and form a leading edge over the wound surface. The mesenchymal connective tissue contained laminin, fibronectin, and various types of collagen. The authors opined that the amniotic membrane provided physical coverage over the wound bed. Processed amniotic membrane shared structural similarities with dermal basal membrane, enabling epithelial keratocytes to migrate effectively through the scaffold.

Pontes *et al.* (2014) compared and studied outcomes of penetrating keratoplasty using bovine amniotic membrane and n-butyl 2- cyanoacrylate. The authors noticed that in the groups that received the amniotic membrane alone, the amniotic membrane acted as a scaffold and aided in superior repair. Histological evidence confirms the migration of endothelial cells on the amniotic membrane. The authors also proposed that the combination of amniotic membrane along with tissue adhesive provided greater corneal repair than using it alone.

Favaraon *et al*. (2015) reported that the amniotic membrane comprises three fundamental layers: the epithelial layer, the basal layer, and the mesenchymal layer, irrespective of the species. These layers play a vital role in maintaining structure and function of amniotic cells.

According to Stachon *et al.* (2015), the precise mechanism by which amniotic membrane enhanced corneal healing remains uncertain; studies indicated that it could be attributed to the interactions of cytokines, growth factors, and protease inhibitors.

Al-Falahi *et al.* (2017) compared the healing effects of bovine amniotic and bovine pericardial membranes in rabbit wounds. Authors opined that the amniotic membrane served as a biomaterial for wound healing; both bovine amniotic membrane, and pericardial membrane showed beneficial effects in the healing of rabbit wounds.

Capistrano da Silva *et al*. (2021) reported from their ex-vivo corneal healing studies in rabbits that the bovine amniotic membrane had a tremendous effect in speeding up the rate of corneal epithelial restoration. Authors emphasized that as a potent therapy to improve stromal and epithelial healing.

2.6. PROCESSING OF BOVINE AMNIOTIC MEMBRANE

Park *et al*. (2008) collected amniotic membranes from healthy placenta, disinfected using 70% ethanol and 0.05% sodium hypochlorite. The epidermis was removed, and the stromal layer was treated with 0.25% trypsin, 0.025 EDTA, and 0.9% NaCl at pH 7.4 for 60 minutes to remove the cellular components. The acellular AMs were dried to maintain their biochemical and structural properties. Individual AMs were sterilized with 25 kGy of gamma irradiation and stored below room temperature until further use.

Said *et al*. (2009) reported that after processing and preservation of the amniotic membrane, the epithelial and stromal cells in the amnion were rendered nonviable. The authors also reported that even though epithelial cells were visible in the thawed membrane, the stroma was particularly devoid of cells, and practically no DNA could be extracted from the amniotic stroma.

Al- Falahi *et al*. (2017) opined that sterile amniotic membranes obtained from post-partum cows can be decellularized in a mixed solution containing 0.1% acetic acid and 4% ethanol for 2 hours. The resulting materials were rinsed with

Phosphate Buffered Saline (PBS) and deionized water for 15 minutes. Further storage was done at 4 ^{0}C in PBS along with 1% gentamicin.

Ballosteros *et al*. (2020) reported that the bovine amniotic membrane could be decellularised by keeping the amniotic membrane in liquid nitrogen (-196 ^{0}C) for 22 hours, followed by a thawing cycle for 2 hours at 37 ^{0}C. Subsequently, they were treated with both strong and weak detergents, sodium dodecyl sulfate (SDS) or Tween 80 for 4 hours. This was followed by immersion in a base solution (0.1M NaOH) for 1 hour and then an acid solution (a combination of peracetic acid and ascorbic acid). As a final step, membranes were washed in 70 percent ethanol for 1 hour to remove residual nucleic acids and phospholipids, followed by a two-hour rinse with phosphate-buffered saline solution. Throughout the procedure, membranes were mechanically stirred in an orbital shaker to ensure thorough washing while minimizing damage to tissue ultrastructure.

Capistrano da Silva *et al*. (2021) studied rabbit ex-vivo corneal healing using bovine amniotic membrane homogenate. The Authors stated that the bovine amniotic membrane underwent three washes for a period of three hours with sterile phosphate buffered saline and an antibiotic-antimycotic solution. Subsequently, the membranes were aseptically cut into pieces and kept in a solution containing 50 microgram/mL gentamicin and Hank's balanced salt solution (HBSS). Until further use, the membranes were stored at -80 ^{0}C and were used after thawing at room temperature.

2.7. SURGICAL TECHNIQUE INVOLVING AMNIOTIC MEMBRANE

Kruse *et al*. (1999) reported the efficacy of multilayer amniotic membrane transplantation in addressing deep ulcers that were unresponsive to conventional treatments. The authors filled the defect with amniotic membrane pieces followed by overlaying them with an amniotic membrane and securing it to the cornea through suturing. The authors noted epithelialisation, reduction in ocular inflammation, and restoration of corneal stromal thickness.

Su and Lin (2000) treated corneal perforations with a combination of amniotic membrane and tissue adhesive. The authors inserted a 1.5 mm section of amniotic membrane directly under the perforation in the anterior chamber and applied cyanoacrylate adhesive over the perforation.

Letko *et al*. (2001) studied inlay and overlay approaches using amniotic membranes for treating persistent corneal defects. The authors noticed no disparity between the inlay and overlay techniques considering the recurrence rate of defects and duration taken for healing.

John *et al*. (2002) reported that toxic epidermal necrolysis could be successfully managed by grafting with a human amniotic membrane. The Authors surgically grafted sections of human amniotic membrane over the affected eyes. Membranes were fixed around the limbus of the eye using multiple 10-0 vicryl sutures.

Dua *et al*. (2004) stated that 10-0 nylon, as well as 8-0 to 10-0 vicryl, are the commonly used suture materials for securing the amniotic membrane over the cornea. Interrupted, running, or mattress types of sutures are the preferred suture patterns. Specifically, mattress sutures are placed tangentially to the limbus, securing it to the episclera or the superficial sclera.

Tseng *et al*. (2004) reported that amniotic membrane can be used for various ocular surface reconstruction procedures as a long-term or temporary solution. In permanent applications, surrounding host cells migrated into the amniotic membrane. In temporary applications, the amniotic membrane serves as a patch or dressing, allowing the host cells to migrate underneath it. After complete healing membrane dissolves naturally.

Non-healing corneal ulcers can be managed by the application of a single layer of amniotic membrane sutured over the ulcer to cover the defect. But in corneal thinning, descemetocele, and corneal perforations, multiple layers of amniotic membrane were used to fill the depth of the defect. Grafts were placed with their epithelial side facing up and sutured with 8-0 to 10-0 Vicyl or Nylon.

Chemical burns, spheroidal degeneration, bullous keratopathy, etc are managed by superficial keratectomy with amniotic membrane transplantation (Thatte, 2011).

2.8. CORNEAL HEALING

Kamiyama *et al*. (1998) reported that platelet-derived growth factor (PGDF) isoforms, including PDGF-AA, PDGF-BB, PDGF-AB, and particularly PDGF-AB, were found to promote the migration of rabbit corneal epithelial cells. The authors noticed this effect when fibronectin, which constituted a part of the provisional extracellular matrix, was found at the wound site.

Jester *et al*. (1999) reported that the corneal epithelium heals through-migration, mitosis, and differentiation. The authors described the course as follows: when the injury extends up to the corneal stroma, a healing process is initiated with apoptosis of keratocytes near the wound site, followed by activation, transformation, and migration of keratocytes from surrounding areas. These activated keratocytes adopt a fibroblast-like phenotype and begin secreting the extracellular matrix. Subsequently, some of the fibroblasts differentiated into myofibroblasts, which have contractile properties.

Kruse *et al*. (1999) opined that corneal perforation and stromal melting can be treated by filling the defects with multilayer of amniotic membrane transplants. The authors reported that the membrane served as a scaffold for keratocytes to migrate and rebuild stromal tissue effectively. The authors also noted that collagen arrangement in these regenerated areas differs from that of normal cornea.

Ma and Bazan (2000) noticed that PAF or platelet-activating factor, which is a bioactive lipid compound, appeared in the cornea following injury. The presence of PAF triggers various biochemical reactions linked to inflammation and the healing process. PAF quickly activated phospholipase A2, leading to the production of arachidonic acid and specific production of prostaglandins in the corneal epithelium. It further encouraged the production of COX-2, which synthesized prostaglandins associated with the inflammatory response.

In a study by Chandrasekhar *et al*. (2001), the authors reported that in organ-cultured rabbit corneas, keratinocyte growth factor (KGF) accelerated the healing of corneal epithelial wounds. Apart from this, KGF was found to boost cell proliferation in the limbal epithelium.

Multiple cytokines, including IL-1, TNF-α, bone morphogenetic proteins BMP-2 and 4, epidermal growth factor, and platelet-derived growth factor, are released from the injured corneal epithelium and epithelial basement membrane. These cytokines contributed to cell migration, cell proliferation, and cell adhesion, as documented by Wilson *et al*. (2001).

Gris *et al*. (2002) noticed that when amniotic membranes were used to correct stromal defects, the membrane initially filled the defect and later got substituted by fibroblasts and collagen.

Wollensack *et al*. (2007) reported from their studies on corneal collagen crosslinking with riboflavin and UV-A in rabbit models that epithelial closure occurred within 3-7 days. Authors noted mild inflammatory infiltrate consisting of polymorphonuclear leukocytes, lymphocytes, and macrophages. Stromal oedema resolved after one week, with keratocyte repopulation and mitoses. Endothelial layers were found to be intact after 7 days. All these suggested a rapid healing of corneal injuries in rabbit models.

Said *et al*. (2009) stated that cells originating from the corneal stroma can migrate and populate the amniotic stroma in significant quantities. These observations suggested that the amniotic stroma offers a supportive structure and environment for the repopulation of stromal cells. The stromal cells represented the myofibroblastic and fibroblastic differentiation of keratocytes.

Lu *et al*. (2010) noticed that corneal cells express various growth factors and cytokines during wound healing exerting specific effects on corneal epithelial cells. These included epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor- α and β (TGF-α, and TGF-β), acidic and basic fibroblast growth factors (FGF-1 and FGF-2), insulin-like growth factor-1 (IGF-1),

keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), Interleukins-1,6, and 10 (IL-1, IL-6, and IL-10) and tumor necrosis factor-α (TNF-α). Authors reported that activation of many of these factors occurred during the wound healing process.

Corneal epithelial wounds healed through coordinated actions such as cell migration, proliferation, adhesion, and differentiation, resulting in the stratification of cell layers. This process relied on interactions mediated by growth factors, cytokines, and signals from the extracellular matrix at the wound site. These interactions aided in regaining epithelial integrity and normal function (Ljubimov and Saghizadeh, 2015).

2.9. CLINICAL SIGNS OF CORNEAL INJURY

Gum *et al*. (1999) noticed that disruptions in the rabbit corneal epithelium caused symptoms like tearing (epiphora), involuntary spasms of the eyelid muscle (blepharospasm), redness of the conjunctiva (conjunctival hyperemia), and inflammation of the anterior part of the eye (reflex anterior uveitis). The authors also reported that this uveitis would be manifested as pupillary constriction and the presence of proteins in the fluid of the eye (aqueous flare).

Andrew (2002) reported epiphora (excess tearing) as a clinical indication of corneal disease. Corneal diseases can also result in corneal opacity due to factors like edema and scarring, as well as the development of new blood vessels (neovascularisation).

Mandell and Holt (2005) reported the clinical indicators of corneal trauma as an involuntary spasm of the eyelid muscles, redness of the conjunctiva, ocular discharge from the affected eyes, corneal oedema, and constriction of the pupil. Authors opined that infected ulcers typically presented with a yellow-white infiltration into the corneal tissue, and deep corneal ulcers would have evident corneal neovascularization.

2.9.1. Presence and Nature of Ocular Discharge

Okud and Campbell (1974) noticed that purulent discharge with redness of the conjunctiva indicated conjunctivitis and infections of the nasolacrimal duct. The authors opined that the diagnosis can be solely confirmed by culture specimens from the conjunctival sac in which *Pasteurella* species were rabbits' most common bacterial pathogens.

Williams (2007) stated that ophthalmic emergencies rabbits are characterised by watery discharge from the eyes, involuntary spasms of the eyelid muscles, and swelling of the eyes and eyelids. Ocular discharge resulted in matting of the lateral as well as medial canthus of the eye. The authors recommended collecting corneal swabs for culture and sensitivity since rabbits are prone to bacterial diseases caused by *Pasteurella multocida, E. Coli,* and various staphylococcal species.

2.9.2. Nature and Number of Blinks Per Minute

Evinger *et al*. (1984) stated that photographic evidence of blinking in rabbits and guinea pigs revealed that the upper eyelid moved downward and outward. The authors reported that in blinking, orbicularis oculi played an active role and levator palpebrae played a passive role primarily.

According to Peiffer *et al*. (1994), the typical blinking rate in rabbits is low, usually averaging around 10 to 12 blinks per hour.

2.9.3. Conjunctival Changes

Danjo *et al*. (1987) stated that conjunctival epithelium underwent cell division in response to injury and contributed to the healing process in acute corneal epithelial wounds. The authors proved these through the changes in the rate of cell division in the epithelium and variation in the content of goblet cells.

Shin and Lee (2006) reported that LASIK surgeries in rabbit models using suction rings lead to inflammatory response and conjunctival apoptosis. The authors

opined that whenever there is tissue damage in the cornea or associated structures it could contribute to the occurrence of cell death in the conjunctiva.

2.10. OPHTHALMOSCOPIC EXAMINATION

According to Kern (1997), assessing the eyes of rabbits and rodents could pose challenges due to their small eye size, challenges in restraint, and the need for larger conventional diagnostic tools.

The smallest aperture on the direct ophthalmoscope is ideal for inspecting the anterior chamber to detect aqueous flare. For a thorough examination of the lens and posterior segment, dilation of the pupil can be achieved by using 1% Tropicamide or a mixture of 1% Atropine and 10% Phenylephrine. The animal should be elevated to evaluate the posterior segment, and the examiner should look upward to visualise the optic nerve (Andrew, 2002).

According to Ollivier (2003), corneal examinations using ophthalmoscopy were preferred to evaluate loss of transparency, vascularisation, pigment deposition, abnormal growths, lacerations, foreign bodies, contour change, and ulcerations.

Mitchell (2011) suggested the procedure for direct ophthalmoscopy as, after adjusting the light intensity to a minimum using a rheostat to avoid the patient's discomfort, the examination could be done in such a way that the right eye to examine the patient's right eye and vice versa. Begin by obtaining the tapetal reflection from a short distance to align the image. Start by identifying and examining the optic nerve head, followed by examining tapetal and non-tapetal quadrants, vascular patterns, corneal clarity, etc.

2.10.1. Corneal Clarity

Corneal stroma plays a crucial role in the development of corneal haze following injury. After a corneal injury, corneal oedema resulted in a corneal haze. Haze arising after the initial phases was due to disorientation of the collagen fibrils and accumulation of various macromolecules, including proteins, glycosaminoglycans, and lipids (Moller- Pederson, 2004).

Samuelson (2013) reported that corneal transparency is maintained by the absence of blood vessels, pigment, and myelin in the corneal structure, as well as the organized arrangement of collagen fibrils in a slightly dehydrated state. The author opined that conditions such as diseases, injuries, or surgical interventions can disrupt this transparency.

Sanches *et al*. (2016) developed Royal Veterinary College Corneal Clarity Scores (RVC-CCS), producing reliable reproducibility among observers. The authors found this suitable for evaluating the efficacy of medical or surgical treatments impacting central cornea in small animals. Gradings were ranged from G0 to G4. The scoring system proved effective for assessing lesions in the central cornea but was less optimal for lesions in the peripheral cornea- as the iris obstructed the reflection of the fundus during the evaluation of the peripheral cornea.

2.10.2. Corneal Oedema and Vascularisation

Philipp *et al*. (2000) observed that after corneal injury, corneal epithelial cells, endothelium, stromal keratocytes, immune cells like T-cells, and macrophages released pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF). The authors reported that these vascular endothelial cells secreted enzymes that broke down the vascular basement membrane and the surrounding extracellular matrix of the cornea, allowing them to migrate into the corneal stroma. As they move towards the inflammation site, attracted by chemotaxis, these cells proliferate and form new blood vessels with associated branches.

Tsai *et al.* (2000) stated that corneal neovascularisation was managed surgically by limbal stem cell transplantation, amniotic membrane transplantation, laser therapy, and photodynamic therapy. Similarly, drug treatments included steroids, non-steroidal anti-inflammatory drugs, prolactin, angiostatin, and cyclosporin A.

Chang *et al*. (2001) noted that several angiogenic agents were involved in neovascularisation, and these agents included various growth factors and cytokines, such as b-FGF, VEGF, IL-8, and selectin E. The authors also reported about angiostatic molecules, such as thrombospondin, angiostatin, and platelet factor 4, which inhibit vascularisation.

Burri *et al*. (2004) reported that angiogenesis is the sprouting of new vessel segments and follows specific patterns. The authors opined that the basement membrane will be degraded, endothelial cell proliferation, solid sprouts of endothelial cells connecting a neighboring vessel, and restructuring of the sprout into a lumen lined by endothelial cells and formed as a vascular network.

Corneal neovascularization was regulated by local pro-angiogenic and anti-angiogenic factors, and the equilibrium between these factors typically maintained corneal avascularity. Corneal neovascularization was triggered by increased expression of angiogenic cytokines in response to inflammation or hypoxic conditions (Azar, 2006).

Costagliola *et al*. (2013) reported that the metabolic pump within the endothelium was the primary factor affecting corneal deturgescence. The pump facilitated the movement of fluid out of the corneal stroma into the aqueous humor through active transport mechanisms. The process relied on oxygen and ATP for energy; lack of any of these could lead to corneal oedema.

Brooks *et al.* (2017) mentioned neovascularisation of the cornea as a normal phenomenon and a reparative response to injury of the corneal surface.

2.11. NEURO-OPHTHALMIC TESTS

2.11.1. Pupillary Light Reflex

Clarke *et al*. (2003) defined pupillary light reflex as the narrowing of the pupil triggered by an increase in light on the retina. Direct PLR occured when the pupil in the same eye constricted as that stimulated with light. Conversely,

consensual PLR involves the pupil in the opposite eye, constricting light stimulation.

Maggs (2008) explained PLR as not an indicator of vision but merely a subcortical response. Whenever there was physical obstruction of iris, iris atrophy, and excitement due to adrenaline rush in animals there were reduced response or no response at all.

Adhikari *et al*. (2015) defined Pupillary Light Reflex (PLR) as a physiological measure of the nervous system's normal or abnormal functioning. The symmetry of the PLR in response to stimulation of either eye, owing to the decussation of pupillary fibers, presented an opportunity to compare the pupillomotor drive in both eyes.

Warnefors *et al*. (2019) observed that the direct pupillary light reflex was slower and less pronounced in rabbits than in dogs. The consensual pupillary light reflex observed was feeble and had limited diagnostic significance in rabbits. This could be attributed to the fact that only 10% of the optic nerve fibres decussate in rabbits.

2.11.2. Dazzle Reflex

The Dazzle or photic blink reflex can be activated by shining a bright light onto the back of the eye and noting the resulting blink response. The reflex operates at a subcortical level, with the highest reflex centre believed to be the rostral colliculi. The facial nerve serves as an efferent pathway for this reflex. Gelatt (1997).

According to Martin (2001), for a dazzle reflex, there should be an intact retina, optic nerve, optic tract, optic chiasma, supraoptic nuclei, and rostral colliculi. It was explained as the bilateral narrowing of the palpebral fissure when a very bright light stimulates the retina.

Mandell and Holt (2005) reported that menace and dazzle reflexes were valuable for evaluating the retina and optic nerve. The authors also noticed that this is challenging in animals having pain due to partial closure of eyes.

Maggs (2008) opined that the dazzle reflex was triggered by sudden exposure to intense light, prompting a swift blinking reaction and sometimes reflexive movement to turn the head away. Reflex operated on a subcortical level and served as a tool to differentiate cortical blindness from blindness caused by damage elsewhere on the visual pathway, whereas the dazzle reflex would be absent if the damage is complete.

2.12. SPECIAL DIAGNOSTIC PROCEDURES

2.12.1. Schirmer Tear Test

Ochipinti *et al*. (1988) stated Schirmer teat test as a diagnostic aid to rule out keratoconjunctivitis sicca. General clinical use of the test involved placing a standard notched filter paper into the lower conjunctival fornix of the eye and reading the wetness of the paper after one minute.

Abrams *et al*. (1990) reported tear production measurements in rabbits. The mean standard deviation (SD) STT value in normal rabbits was found to be 5.30± 2.96 mm/min (with a range of 0-15 mm/min), and significant differences were noticed among breeds.

Birick *et al*. (2005) observed that the Schirmer tear test is suitable for rabbits and guinea pigs, and the test strip is too large for rats and mice. Significant breed-wise differences were also evident, with Netherland Dwarf rabbits having an unusually high value of 12 ± 2.5 mm/min compared to an average of 5.3± 2.9 mm/min with other breeds.

Petznick *et al*. (2011) defined STT I, which was performed without the use of topical anesthesia, provided a measure of both basal and reflex tearing, whereas STT II, which was performed after topical anesthesia with a drop of tetracaine, demonstrated basal tearing alone. Basal tears were those produced in the absence

of stimulation and were lacrimal secretions that maintained the ocular surface. Reflex tears were produced after stimulation, usually as a result of irritation to the surface, such as a foreign body or an inflammatory response. The tear strip itself likely acted as a source of irritation to increase tear production in STT 1.

Lima *et al*. (2015) reported that qualitative tests used to assess tear film include the Schirmer tear test (STT), Phenol Red tear test (PRTT), and Endodontic absorbent paper point tear test (EAPTT). In New Zealand white rabbits, normal tear test results noticed were approximately 4.8 ± 2.9 to 5.3 ± 2.9 mm by STT, 20.8 ± 3.7 mm by PRTT, and 13.8 ± 1.5 mm/min by EAPTT. Comparatively, tear production values obtained from English Angora and Dutch rabbits were similar to those of New Zealand white rabbits. Results of PRTT and EAPTT indicated higher tear production in Angora and Dutch rabbits.

Maggs *et al*. (2017) noted that lagomorphs Schirmer tear test results were lower than felines. Despite rabbits blinking only in every five to six minutes, their tear film remains relatively stable, and they rarely suffer from exposure keratitis due to evaporation. Tear film stability in rabbits was achieved by the presence of the lacrimal gland, accessory lacrimal gland, superficial gland of the third eyelid, and the harderian gland. Additionally, the lower Schirmer tear test result in rabbits compared to felines can be attributed to the retention of the tear film due to the absence of superior lacrimal puncta.

Variations in STT I results may also result from factors beyond tear production, such as the emotional state of the animal during the examination, differences in Schirmer strips used, and variations in ambient humidity levels within the testing environment (Yoon *et al*., 2020)

Corsi *et al*. (2022) opined that evaluating the quality and quantity of tears in veterinary medicine is crucial for diagnosing and managing ocular conditions. The most common methods used were the Schirmer Tear Test (STT), tear film osmolarity measurement, interferometry, tear meniscus height measurement, and

Meibography. Each method provided valuable information about tear film quality, quantity, and ocular surface health.

2.12.2. Fluorescein Dye Test

Bedford (1982) noted that after taking up the fluorescein stain corneal stroma appears brilliant green under cobalt blue light during the course of a corneal epithelial defect.

Foulks and Pavan-Langston (1985) opined that fluorescein has been utilized in clinical practice to identify corneal epithelial abrasions associated with different types of punctate keratitis or epithelial defects.

Feenstra and Tseng (1992) used fluorescein staining while using amniotic membrane to treat corneal surface irregularities. The authors noticed that fluorescein dye has superior penetration capabilities compared to lissamine green through the corneal stroma.

Corneal stroma, which is hydrophilic, can only retain water-soluble fluorescein dye (Wilkie and Whittakkar, 1997).

Korb (2000) stated that sodium fluorescein and rose bengal dyes have served as the primary dyes for assessing ocular surface disturbances. Fluorescein is recognized as the primary dye for corneal staining, and rose bengal is the primary dye for conjunctival staining. These dyes are extensively used in clinical settings to diagnose conditions related to tear film and dry eye syndrome.

According to Miller (2001), a Fluorescein dye test is performed by instilling a drop of normal saline over an impregnated strip, and diluted dye is allowed to contact the superior bulbar conjunctiva. After allowing the animal to blink the excess stain is washed off for examination.

Costa *et al*. (2019) studied the re-epithelialization of the cornea after grafting corneal ulcers with an amniotic membrane. The author confirmed corneal re-epithelialisation through a negative fluorescein dye test

2.13. CULTURE AND ANTIBIOTIC SENSITIVITY

Every corneal ulcer would undergo scraping and direct inoculation onto culture plates. These plates would then be maintained under suitable conditions in a microbiological laboratory without further processing. If the predetermined criteria for empiric therapy were satisfied, treatment could be initiated accordingly. If the ulcer shows no improvement the interpretation of culture and sensitivity is important (Macleod *et al*., 1996).

Beegum *et al*. (2006), in a study of bacterial isolates from corneal specimens, reported that from the positive cell cultures, majority were attributed to Gram-positive bacteria, with coagulase-negative Staphylococci being the most prominent, followed by Streptococcus pneumoniae, among the Gram-negative bacteria isolated Pseudomonas accounts for positive cultures. All the isolates demonstrated sensitivity to ofloxacin and other ocular antibiotics.

Tolar *et al*. (2006) noticed that corneal culture swabs of dogs showed increased resistance to bacitracin, chloramphenicol, and cephalothin because of the greater prevalence of isolates of *Pseudomonas aeruginosa.* Resistance to fluoroquinolone antimicrobials remained minimal, suggesting ciprofloxacin as the effective treatment for bacterial keratitis in dogs. Tobramycin was preferred over gentamicin due to its lower toxicity to epithelial cells in vitro. On the other hand, resistance of gram-positive bacterial isolates to fluoroquinolones was on the rise.

2.14. HISTOLOGICAL OBSERVATIONS DURING CORNEAL HEALING

Nishida *et al*. (1990) noted that activated keratocytes possess phagocytic properties against foreign materials and play a role in stromal collagen breakdown by producing collagen-degrading enzymes. This mechanism explained the resorption of the amniotic membrane without concurrent stromal vascularisation.

Gris *et al*. (2002) opined that the histopathological examination of the cornea after amniotic membrane grafting revealed initial re-epithelialisation with

5-7 layer thickness of epithelium while partial restoration of stromal thickness. The authors also noted a thin band of eosinophilic infiltrate over the central cornea corresponding to the amniotic membrane graft. Regarding stromal healing, the authors opined that the amniotic membrane initially filled the defect and later got substituted by fibroblasts and collagen

Wang *et al*. (2001) assessed the corneal healing by amniotic membrane in rabbits after excimer photoablation. All corneas were collected using a 9 mm trephine and halved to traverse the ablated centre. Three corneas were preserved in glutaraldehyde 2.5%, subjected to Hematoxyllin-Eosin as well as Masson's trichrome staining, and scrutinised via high-power magnification under light microscopy to evaluate epithelial hyperplasia and stromal fibroblast cellularity.

Dua *et al*. (2004) stated that when used as a patch, the amniotic membrane typically detached prematurely, sometimes before the desired time, or was eventually expelled. Histological examination revealed fibrillar remnants of the amniotic membrane. The membrane's disintegration often manifested as noticeable large gaps beneath the overlying epithelium.

Tosi *et al*. (2005) histologically evaluated corneas after amniotic membrane transplantation and reported that re-epithelialisation, arrangement of newly formed epithelium, presence or absence of amniotic membrane, arrangement of stromal collagen and fibroblasts, presence or absence of PMN cells, neovascualrisation, oedema, etc. are evaluated during the healing process of cornea.

Said *et al*. (2009) reported that when amniotic membranes were transplanted over corneal defects, corneal stroma-derived cells (CSDC) migrate from corneal stroma and repopulate in the amniotic stroma. The authors also reported that during the initial phases of healing, corneal stroma-derived cells extended to the superficial amniotic stroma, while in the later stages, they get repopulated to the deeper layers of the amniotic stroma, providing better acceptance of the graft.

Capistrano da Silva *et al*. (2021) studied the effect of Bovine Amniotic Membrane Homogenate in rabbit ex-vivo corneas by collecting them from the

culture plate on the 7th day, immersed in 10% buffered formalin for 48 hours, moved to an ethanol solution before being embedded in paraffin. Spanning from limbus to corneas were trimmed and then embedded in paraffin. Corneas were horizontally sectioned in a 5 µm thickness and stained with Hematoxyllin and Eosin. On histological analysis, no keratocyte deterioration was evident, and on the healed region, a consistent epithelial layer with basal and superficial cells was observed.

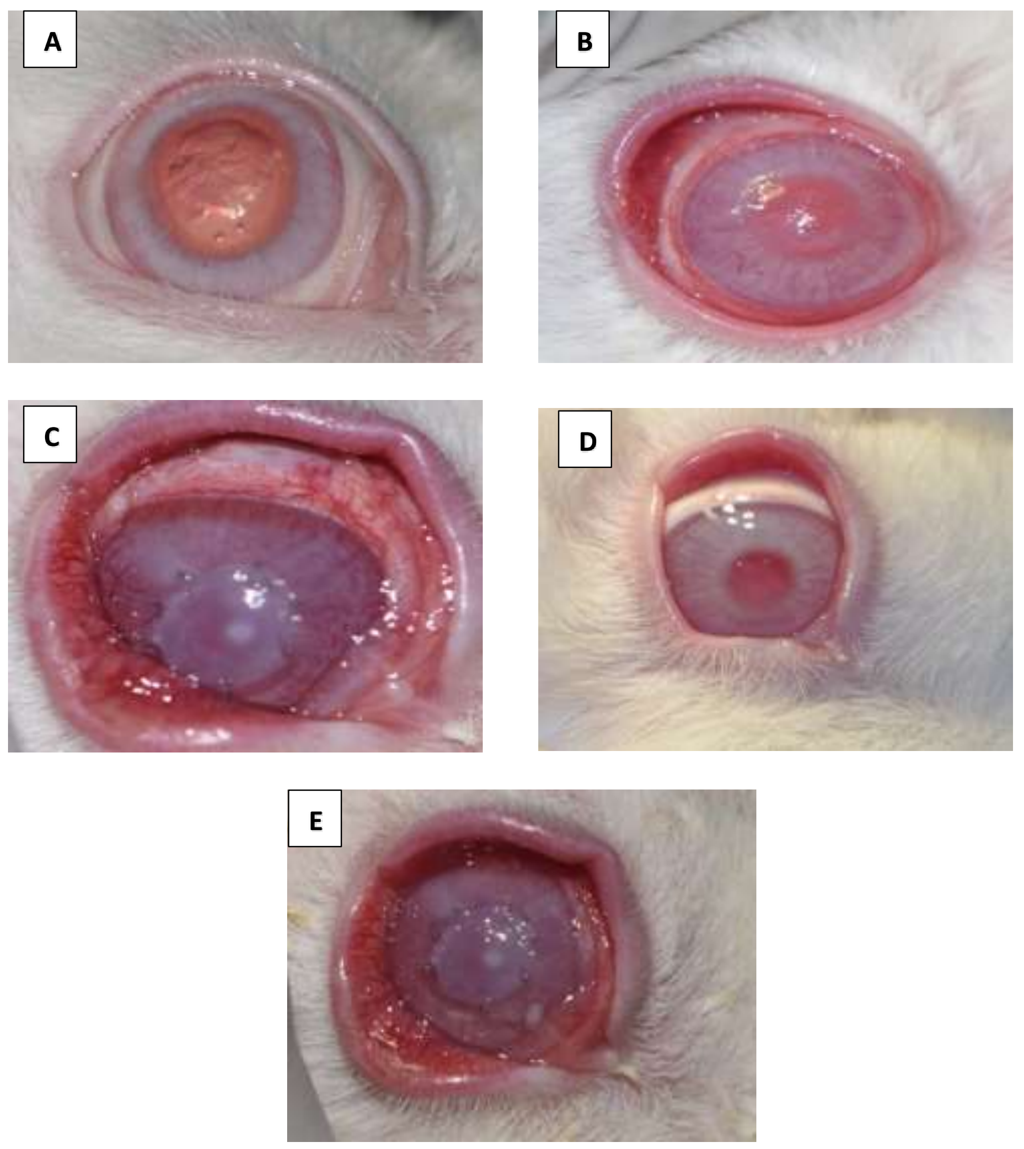


Plate 4 . Conjunctival changes. (A) Normal conjunctiva, (B) Generalised congestion, (C) Injection of vessels, (D) Generalised congestion, (E) Injection of vessels

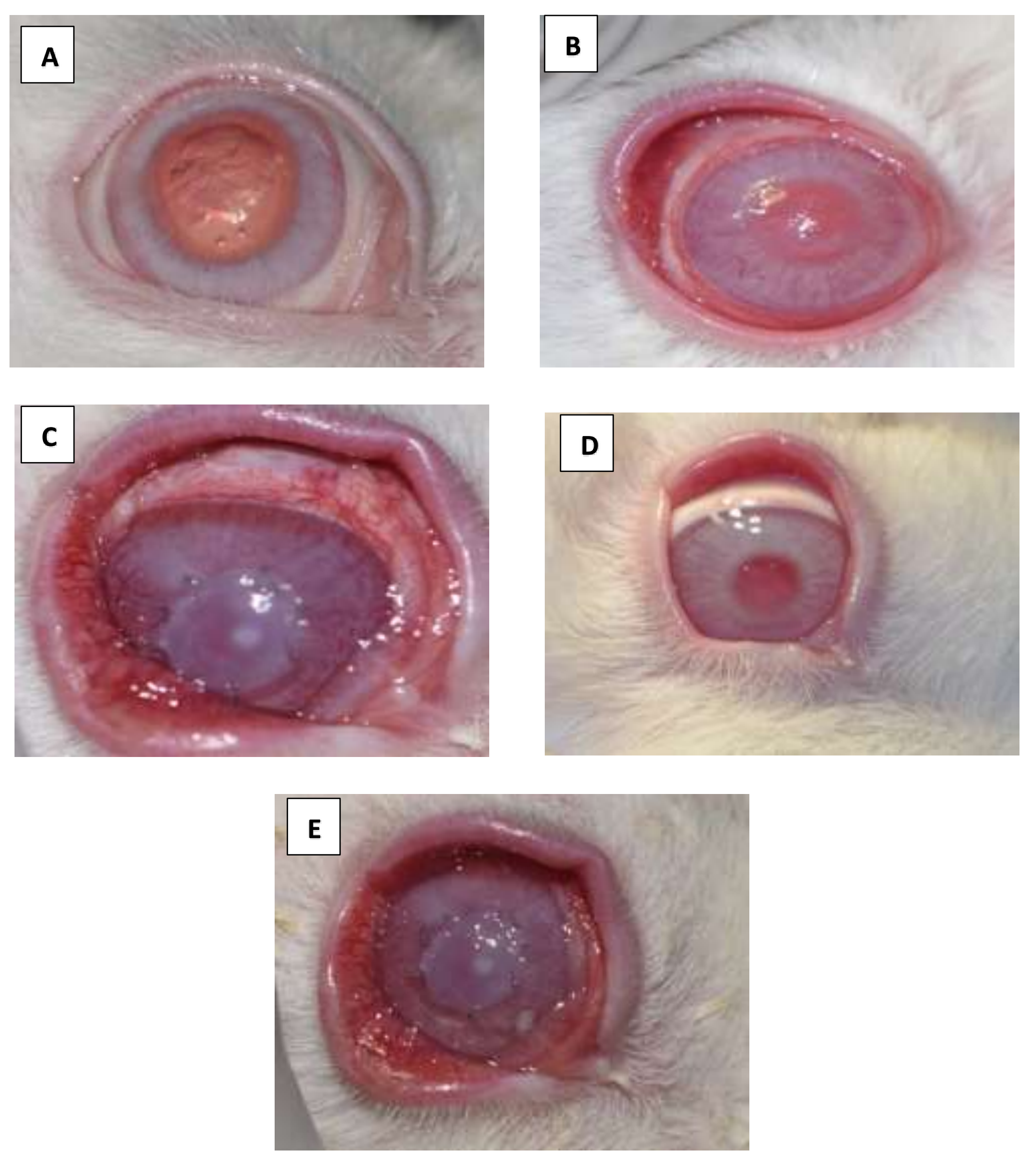


Plate 5. Grading of corneal clarity. (A) Hazy, (B) Hazy, (C) Moderate opacity, (D) Hazy, (E) Moderate opacity

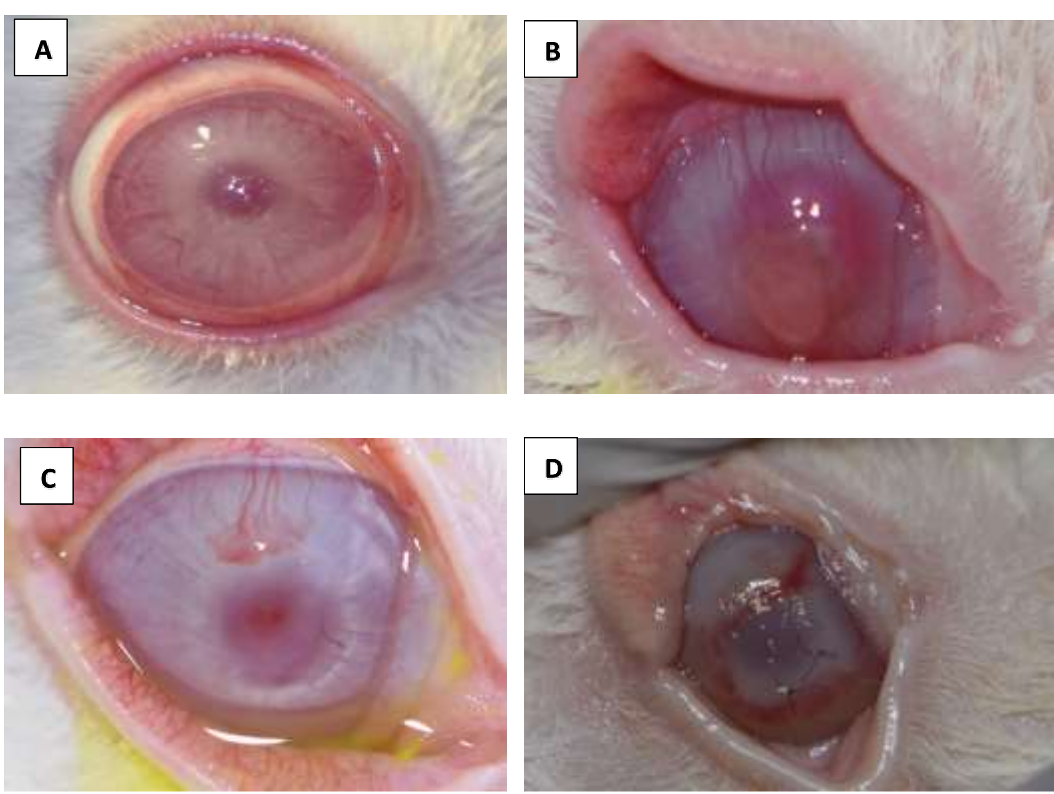


Plate 6. Grading of Corneal oedema. (A) No oedema (Grade 0), (B) Mild oedema (Grade 1), (C) Mild oedema (Grade 1), (D) Marked oedema (Grade 2)

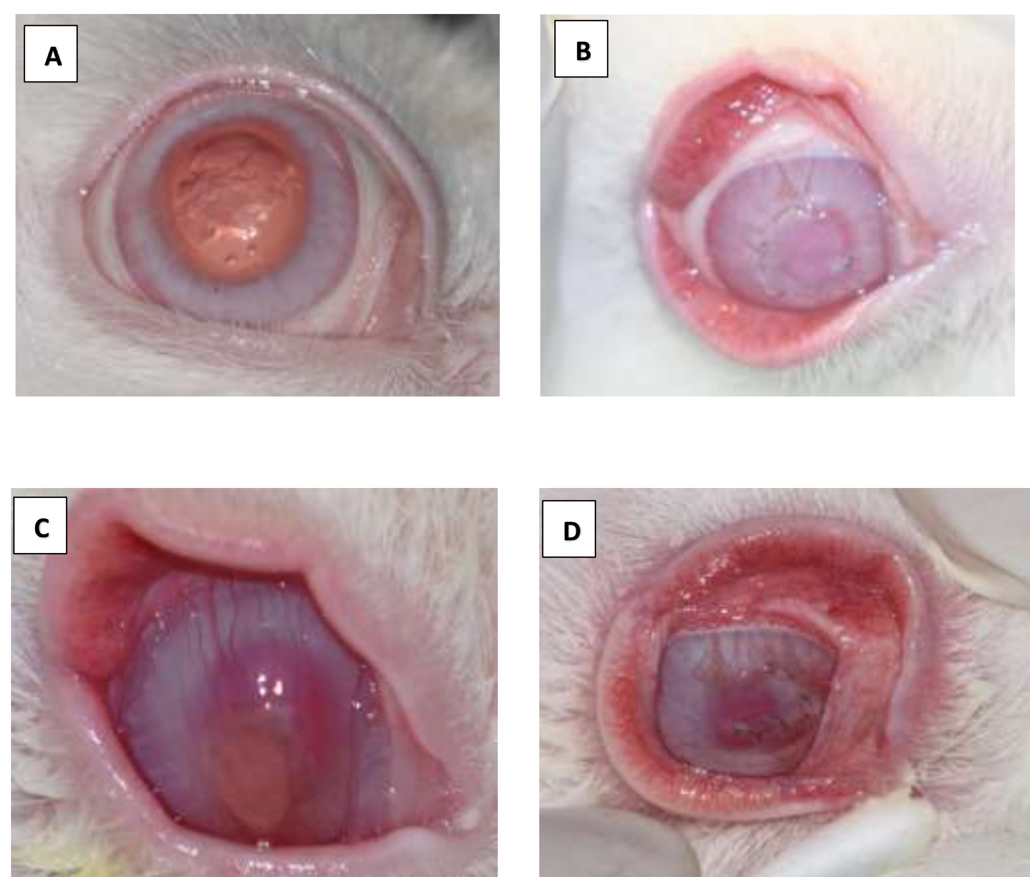
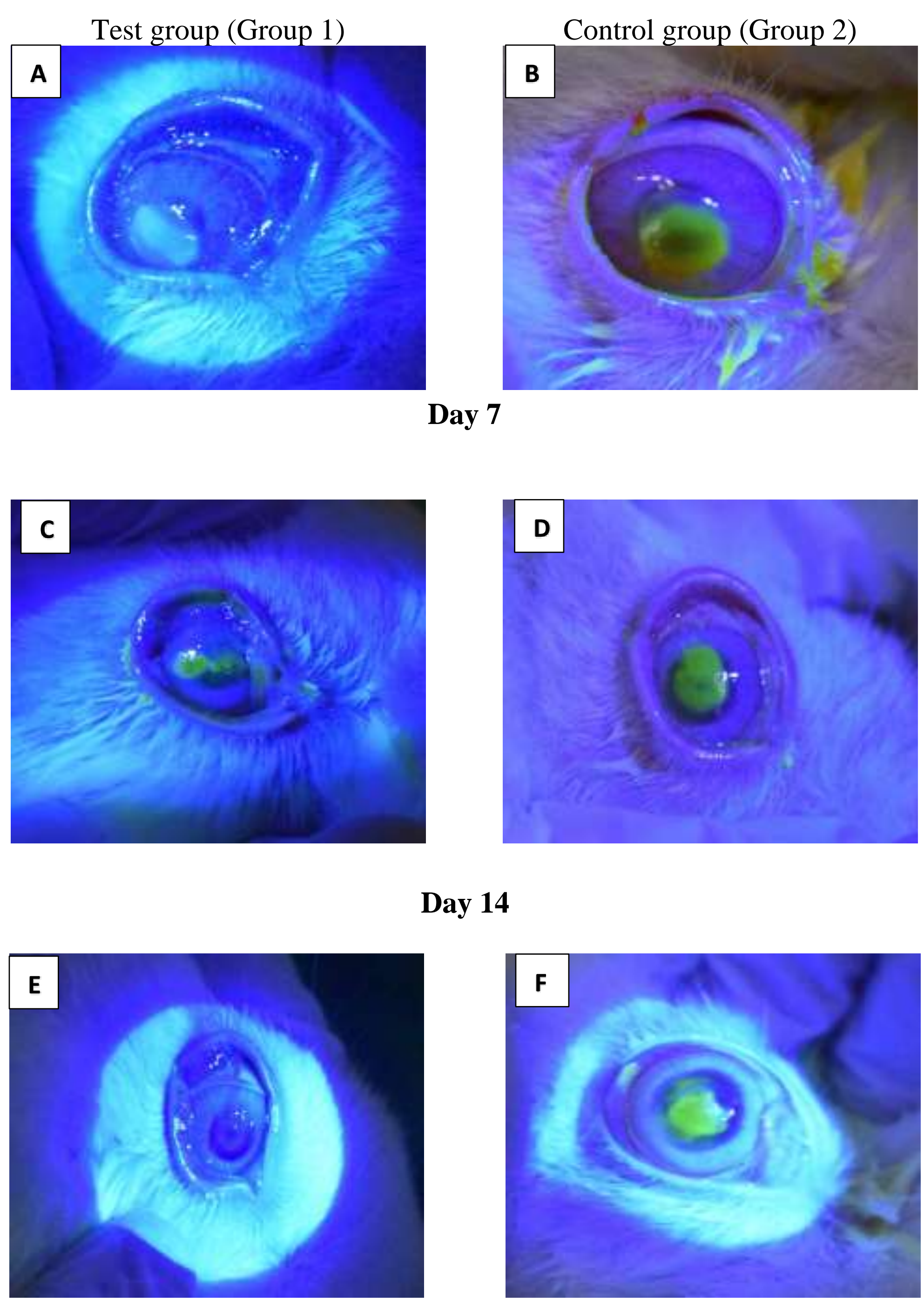
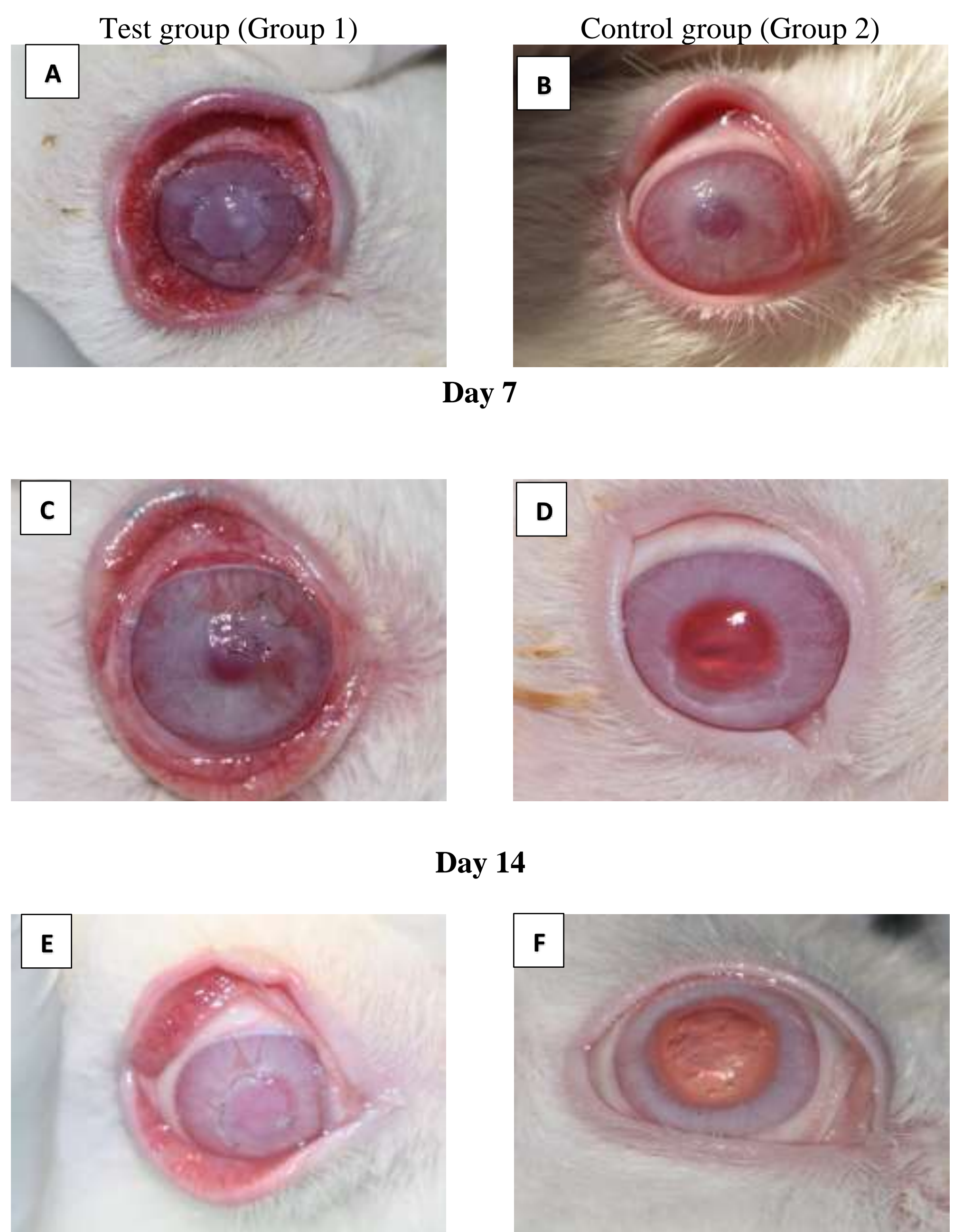


Plate 7. Grading of corneal vascularisation. (A) No visible vascularisation-Grade 0, (B)Mild superficial vascularisation- Grade 1, (C) Profuse Superficial vascularisation- Grade 2, (D) Extensive superficial vascularisation- Grade 3



Day 28

Plate 8. Results of fluorescein dye staining group 1 and group 2 animals



Day 28

Plate 9. Post-operative appearance of cornea in group 1 and group 2 animals

Test group (Group 1)

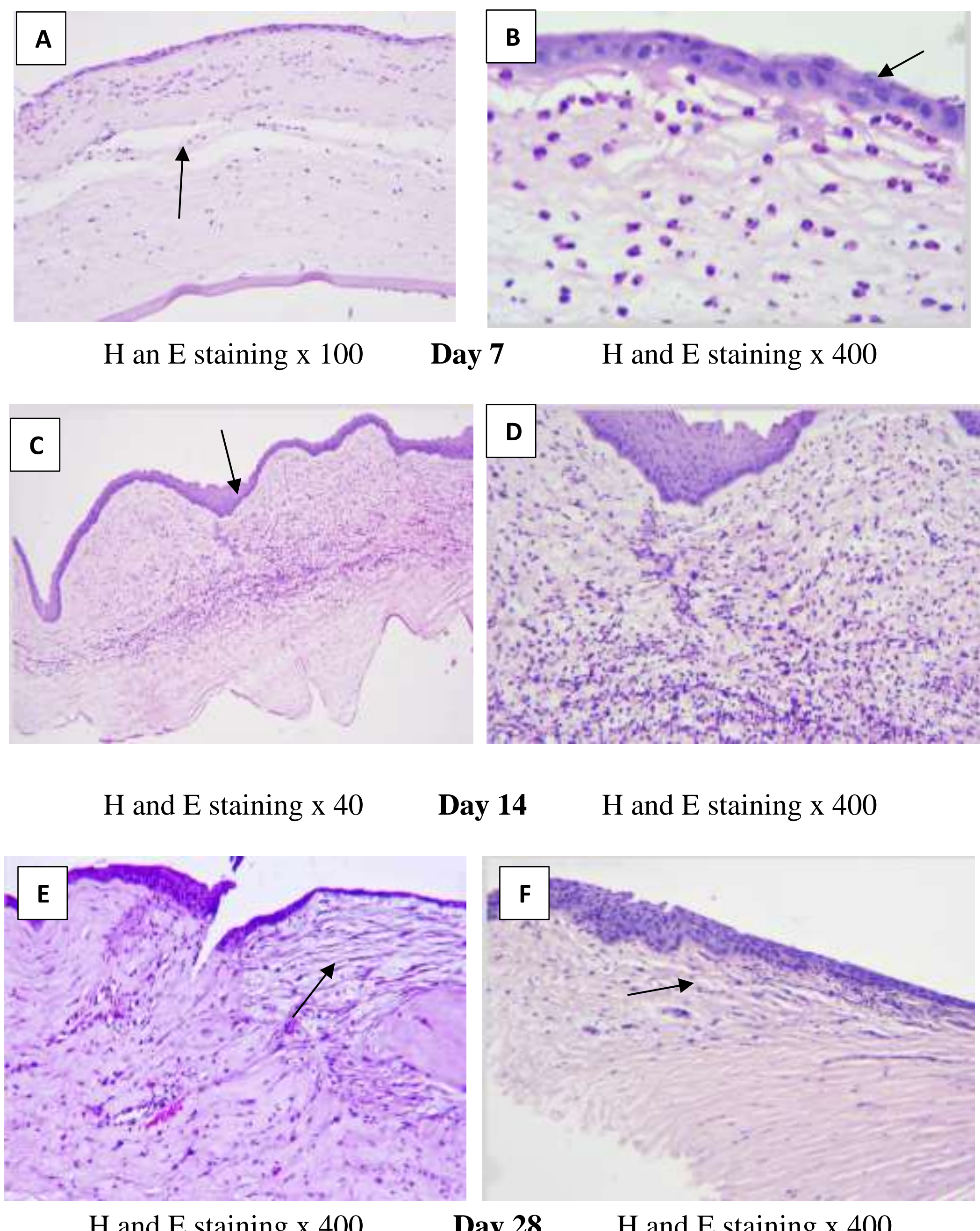


Plate 10. Histological observations in test group (A) Day 7- arrow indicates longitudinal cleft between amniotic membrane and corneal stroma, (B) Day 7- arrow indicate reepithelialisation, (C) Day 14- Arrow indicate reepithelialisation, (D) Day 14-infiltration of inflammatory cells, (E) & (F) Day 28- arrows indicate organised collagen arrangement

Control group (Group 2)

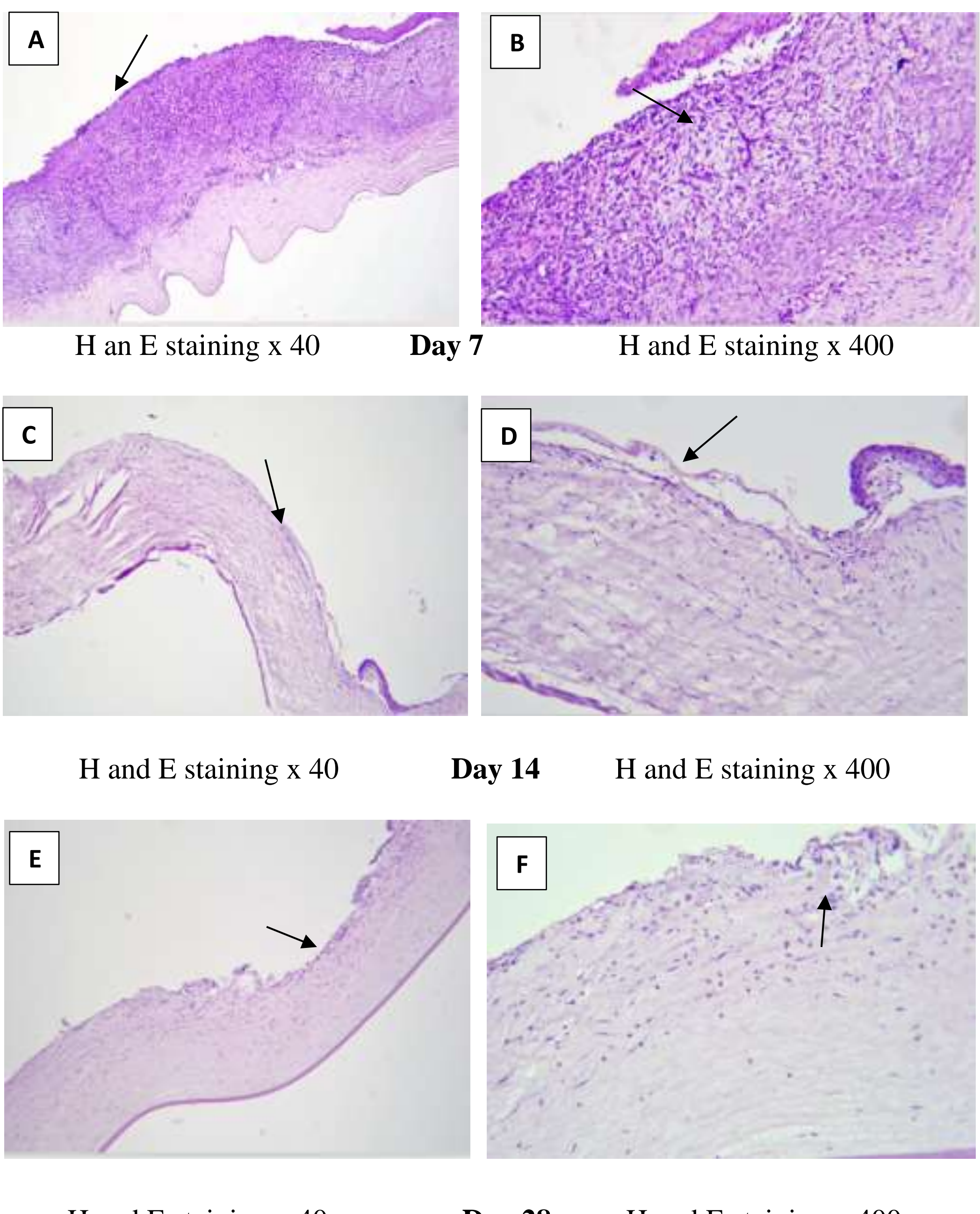


Plate 11. Histological observations in control group. (A) Day 7- arrow indicates reepithelialisation from wound edges, (B) Day 7- arrow indicates infiltration of inflammatory cells, (C) & (D) Day 14- arrow indicates absence of epithelium and disoriented stromal collagen matrix, (E) & (F) Day 28-Arrow indicates absence of epithelium and disoriented stromal collagen matrix

5. DISCUSSION

Corneal wound healing was studied in 18 healthy adult rabbits procured from laboratory animal breeding facility approved by the Committee for Control and Supervision of Experimentation on Animals (CCSEA). The test group (group 1) received decellularized bovine amniotic membrane (BAM) derived extracellular matrix (ECM) scaffold as an inlay graft on the cornea along with topical antibiotic eye drops and anti-inflammatory orally, while the control group (group 2) received topical antibiotic eye drops and anti-inflammatory orally alone. Ophthalmic examinations were done on days 7, 14 and 28 post-surgery, to assess healing of the cornea. The results of the study are discussed below.

5.1. MAIN ITEMS OF OBSERVATION

5.1.1. Presence and Nature of Ocular Discharge

On 7th day of observation, ocular discharge in group 1 was mild mucoid in three animals, mild and serous in two while absent in the rest. In the group 2 animals, ocular discharge was-mild and mucoid in one, moderate and mucoid in one, moderate and serous in one, while absent in the rest. The ocular discharge noticed in the initial days of observation following surgery may be due to the induced corneal wound in both groups. The difference in nature of the discharge may be due to the proliferation of opportunistic bacteria and rise of bacterial load in cornea following injury. Gum *et al*., (1999), Andrew (2002) and Mandell and Holt (2005), stated ocular discharge as the clinical indicator of corneal trauma. On 14th day of observation, ocular discharge in group 1 was mild and serous in two animals while it was absent in the rest. In group 2, ocular discharge was moderate and mucoid in one animal while there was no discharge in the rest. On 28th day of observation, there was no ocular discharge in any of the animals. The absence of ocular discharge in group 1 may be attributed to the anti-bacterial, anti-inflammatory, and reepithelialising properties of bovine amniotic membrane

(Litwiniuk and Grzela, 2014), which helped in complete healing of the corneal defect evident from clinical as well as histological examination. Even though there was absence of complete reepithelialisation of the defect noticed from clinical and histological examination, the reduction of ocular discharge in group 2 could be attributed to the anti- bacterial effects of topical antibiotics.

5.1.2. Results of Visual Function Tests

Pupillary light reflex was sluggish in all the animals, in both the groups, on all days of observation. The findings in the current study was in accordance with Warnefors *et al*., (2019) who reported that direct pupillary light reflex was slower and less pronounced in rabbits compared to that in dogs. Dazzle reflex was positive in all the animals, in both the groups, on all days of observation. Dazzle reflex can be initiated only if the optical axis is transparent and the light source is bright enough. The positive dazzle reflex in the animals studied suggests that the cornea was transparent enough to transmit the bright light to the retina and to the visual cortex. A positive dazzle reflex denotes intact retina, optic nerve, optic tract, optic chiasm, supraoptic nuclei and rostral colliculi as per Martin (2001). Hence the findings from dazzle reflex suggests that vis

5.1.3. Nature and Number of Blinks per Minute

There was no blinking noticed within a minute in any of the animals. According to Peiffer *et al*., (1994), the typical blinking rate in rabbit is low, usually averaging around 10 to 12 blinks per hour.

5.1.4. Conjunctival Changes

On 7th day of observation, there was injection of palpebral conjunctival vessels in all the animals of group 1. In group 2, there was generalised congestion of palpebral conjunctiva in five animals and no changes in the rest. Conjunctival changes noticed in both groups may be due to the induced corneal injury and manipulation of the eyeball during the surgical procedure. The conjunctival changes observed in our study align with the findings of Gum *et al*., (1999), indicating that conjunctival hyperaemia occurs when there is corneal epithelial disruption. The

third eyelid flap, maintained for three days in the test group, may have also caused irritation and inflammation, leading to injected conjunctival vessels. On the 14th day of observation, there was generalised congestion of palpebral conjunctiva in five animals and injected palpebral conjunctival vessels in one animal of group 1. In group 2, conjunctiva appeared normal in all the animals. There was considerable reduction in injection of vessels and congestion. In group 1, most animals showed a transition from injected vessels to generalized congestion, whereas the conjunctiva has become normal in group 2. On 28th day of observation, there was injection of palpebral conjunctival vessels in one animal, and generalised congestion in two animals of group 1. The conjunctival epithelium undergo cell division followed by conjunctival hyperemia in response to injury, aiding in the healing of acute corneal epithelial wounds (Danjo *et al*., 1987, and Shin and Lee 2006). The conjunctival changes happening over the days of observation in group1 could be attributed to the healing process in response to the induced corneal wound. Animals of group 2 were presented with a normal conjunctiva on 28th day of observation. The reduced conjunctival hyperaemia in group 2 suggests that conjunctival epithelium might not have been contributing towards the corneal healing as evident from their clinical as well as histological findings. The reduced manipulation of the globe and conjunctiva during surgery may also have contributed to the minimal conjunctival changes in group 2.

5.1.5. Ophthalmoscopic Examination

5.1.5.1. Corneal Clarity

On 7th day of observation, central cornea was moderately opaque, while the surrounding cornea was clear in all the animals of group 1. The moderate central corneal opacity noticed could be attributed to the texture of grafted bovine amniotic membrane over the created defect. In group 2, the central cornea was hazy while the surrounding area was clear in all the animals. Corneal haze arising after the initial phases of corneal injury could be attributed to the disorientation of the collagen fibrils and accumulation of various macromolecules including proteins, glycosaminoglycans and lipids (Moller- Pederson, 2004 and Samuelson, 2013). On

14th day of observation, central cornea in the animals of group 1 appeared hazy in five and moderately opaque in one. The remnants of the grafted membrane would have contributed to the moderate opacity in one animal. In all the animals, surrounding area of the graft was oedematous with profuse vascularisation noticed from the superionasal and superiotemporal quadrants. The oedema noticed could be attributed to disorientation of collagen fibrils. The profuse vascularisation noticed in the area surrounding the grafted defect suggest healing of the corneal defect as per Brooks *et al*., (2017) who reported neovascularisation as a reparative response to injury of corneal surface. In group 2, central cornea was hazy in all the animals with clear surrounding area. On 28th day of observation, central cornea appeared hazy in all the animals of group 1 and group 2. The surrounding area of the graft was presented with mild vascularisation from superior quadrants in all animals of group 1 while it was clear in all the animals of group 2. The mild corneal vascularisation from superior quadrants evident in group 1 animals on 28th day of observation also suggests the reparative response of cornea following grafting.

In group 1 animals there was marked reduction in the area of haziness observed during the course of the study, while there were no changes in haziness noticed in the central cornea in animals of group 2 throughout the study. The anti-fibrotic property of the grafted bovine amniotic membrane would have contributed to the enhancement of clarity in reducing the area of haziness in group 1. The anti-scarring and antifibrotic effects of the amniotic membrane are due to its ability to prevent fibroblast activation into myofibroblast (Tseng *et al*.,1999).

5.1.5.2 Corneal Oedema and Vascularisation

On 7th day of observation, there was marked central corneal oedema in one animal, mild central corneal oedema in one, and no oedema in the rest of the animals in group 1. The area surrounding the grafted defect appeared clear in all the animals. In group 2, mild central corneal oedema with clear surrounding area was noticed in one animal while there was no oedema in the rest. The corneal oedema observed in both groups may be attributed to water absorption and disarrangement of the normal pattern of collagen fibres of the stroma (Moller-Person, 2004; Costagliola *et al*.,

2013 and Samuelson, 2013). On 14th day of observation, mild central corneal oedema was present in two animals in group 1 and marked central corneal oedema in the rest. In group 2, mild central corneal oedema was observed in four animals while there was no oedema in the rest. There was marked oedematous changes in the surrounding area of the grafted defect in group 1 while no changes were noticed in group 2. The oedematous changes noticed in the surrounding area in group 1 may be attributed to the disorganised collagen fibres of the stroma. The marked oedematous changes noticed in the surrounding area in group 1 animals may be also due to manipulation of eyeball and sutures applied to keep the graft in the cornea. On 28th day of observation, mild oedema was present in all the animals of group 1 and group 2. No changes were noticed in the surrounding areas in both groups. There was transition from marked central corneal oedema on 14th day to mild corneal oedema on 28th day in the group 1 animals. This reduction in corneal oedema noticed group 1 animals may be due to more organised arrangement of collagen fibrils in the stroma towards the 28th day of observation. (Lee and Tseng, 1997 and Tseng *et al*., 1999). There was no change in the oedema in group 2 on 28th day of observation, which may be attributed to the disorientation of stromal collagen matrix lacking normal architecture of parallel arrangement in the induced corneal wound.

On 7th day of observation, mild superficial corneal vascularisation was visible in two animals, while there was no visible vascularisation in the rest in group 1. In group 2, mild superficial vascularisation was noticed in one animal, while there was no visible vascularisation in the rest. Philipp *et al*., (2000), Chang *et al*., (2001), and Burri *et al*., (2004) observed that following corneal injury, corneal epithelial cells, endothelium, stromal keratocytes, immune cells like T-cells and macrophages released pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF) and responsible for corneal neovascularisation. On 14th day of observation, animals in group 1 showed mild superficial vascularisation in three, profuse superficial vascularisation in two, and extensive superficial vascularisation in one. In group 2, mild superficial vascularisation was noticed in one animal while there was no visible vascularisation in the rest. On 28th day of observation, mild

superficial vascularisation was noticed in the three animals of group 1, while there was no visible vascularisation in the animals of group 2. During the course of the study, there was visible vascularisation in group 1 animals on all days of observation while no prominent vascularisation was noticed in group 2 which can be attributed to the finding that there is less reparative response to corneal injury in group 2. The profuse vascularisation noticed in the area surrounding the grafted defect suggest healing of the corneal defect as per Brooks *et al.*, (2017) who reported neovascularisation as a reparative response to injury of corneal surface. Neovascularisation noticed in the present study may also have aided in distributing growth factors which further helped in healing. Even though it was reported that amniotic membrane has anti-angiogenic effects, the anti- angiogenic effects are more evident in fresh amniotic membrane than in preserved. Preserved amniotic membrane possess angiostatic properties, where they retard vascularization by mopping up the diffusion of inflammatory mediators and vascularization promoters (Hao *et al.,* 2000).

5.2 SPECIAL DIAGNOSTIC TESTS

5.2.1 Results of Schirmer Tear Test

The mean ± SE values (mm/min) for STT1 in group 1 animals were 10.22 ± 0.954, 11.50 ± 0.619, 6.33 ± 1.764 on days 7, 14 and 28 respectively. In the group 2 animals, the values on day 7, 14 and 28 was 10.00 ± 0.527, 6.50 ± 0.922, 7.00 ± 1.732 respectively. The STT value in the animals of group 2 on 14th day of observation was significantly lower ($p > 0.01$) than that of animals in group 1. The significant difference could be attributed to the irritation caused by the presence of non-absorbable sutures that have been used to secure the graft over central corneal defect in group 1 animals. According to Abrams *et al.*, (1990), the mean standard deviation (SD) STT value in normal rabbits was 5.30±2.96 mm/min. In the current study, by the 28th day of observation, the mean values in both groups were approaching the normal range, likely due to the anti-inflammatory properties of the amniotic membrane (Lee and Tseng, 1997; Tseng *et al.,*1999; Litwiniuk and Grzela,

2014; Capistrano da Silva *et al*., 2021) in group 1 animals. In group 2 animals, a reduction in STT values was observed on the 14th day, likely due to the combined effects of oral anti-inflammatory and topical antibiotics. However, by the 28th day, there was a subsequent increase in STT values, which may be attributed to the presence of an unhealed defect in group 2 animals.

The mean ± SE (mm/min) values for STT2 in group 1 animals were 5.33 ± 0.471, 6.50 ± 0.922, and 7.33 ± 2.333 on days7, 14 and 28 respectively. In group 2 the means were 3.56 ± 0.626, 2.17 ± 0.477, and 2.67 ± 1.202. The STT values in the animals of group 2 on 7th and 14th day of observation was significantly lower ($p > 0.01$) when compared to the respective values of group 1. The non healed corneal wound in group 2 animals might have reduced the basal tear production leading to significantly lower results in group 2 compared to group 1. The retention of tear film in control animals might have also been aided in significant lower values in group 2 compared to group 1 as reported by Maggs *et al*. (2017) who reported that the lower Schirmer tear test result in rabbits compared to felines can be attributed to the retention of the tear film due to the absence of superior lacrimal puncta.

5.2.2 Fluorescein Dye Test

Fluorescein dye staining was noted in all the animals in group 1 and group 2 on 7th day of observation. On 14th day, fluorescein dye staining was noticed in five animals in group 1, but there was no staining in the remaining one. In group 2, fluorescein dye staining was noticed in all the animals. Corneal stroma which is hydrophilic retains the water-soluble fluorescein dye whereas the lipophilic corneal epithelium does not (Wilkie and Whittakkar, 1997). On 28th day of observation, all animals of group 1 tested negative for fluorescein dye staining. In group 2, all animals tested positive for fluorescein dye staining. The negative dye staining in group 1 could be attributed to the re-epithelialisation of cornea. According to Kruse *et al*., (1999), when corneal perforation and stromal melting defects are filled with multilayer amniotic membrane transplants, the membrane served as a scaffold for

keratocytes to migrate and rebuild stromal tissue effectively. The authors also noted that collagen arrangement in these regenerated areas differed from that of normal cornea. So the amniotic membrane graft applied over the corneal defect may have helped in faster re-epithelialisation in the current study. Cells originating from the corneal stroma can migrate and populate the amniotic stroma in significant quantities. These observations suggested that the amniotic membrane stroma offers a supportive structure and environment for repopulation of stromal cells. Also, cell migration, proliferation and differentiation relies on interactions mediated by growth factors, cytokines and signals from the extracellular matrix at the wound site. All these factors contribute to faster recovery of the corneal epithelium. (Said *et al*., 2009; Lu *et al*., 2010; and Ljubimov and Saghizadeh, 2015).

5.2.3 Culture and Antibiotic Sensitivity of Corneal Swab

Culture of corneal swabs from animals in both groups revealed gram-positive cocci. Majority of the cultures from group 1 and group 2 animals were sensitive to amikacin and gentamicin while resistance was noticed against moxifloxacin, ofloxacin, and ciprofloxacin in both groups. According to Tolar *et al*., (2006), tobramycin is the preferred topical antibiotic over gentamicin due to its lower toxicity to epithelial cells in vitro. Even though many of them were sensitive to gentamicin, but to reduce the toxicity to epithelial cells caused by gentamicin, tobramycin was preferred. The authors also stated that resistance of gram-positive bacterial isolates to fluoroquinolones is on the rise which is evident from the antibiotic sensitivity report.

5.3 HISTOLOGIC OBSERVATION DURING HEALING PROCESS

Histologic observation of the corneas in group 1 animals on 7th day of examination revealed an intact amniotic membrane overlying the corneal defect. A small longitudinal cleft separated the amniotic membrane from the corneal stroma. Two to three layered stratified epithelium was noticed invading from the wound

margins and continuous with the anterior margin of the amniotic membrane. Gris *et al*. (2002) reported that histopathological examination of the cornea following amniotic membrane grafting showed initial re-epithelialization with a five to seven layer thick epithelium and partial restoration of stromal thickness, which aligns with the observations in the current study. Inflammatory cells like polymorphonuclear cells, lymphocytes, macrophages and a few fibroblasts were noticed invading the membrane and the anterior stroma. Ma and Bazan (2000) noticed that PAF or platelet-activating factor- a bioactive lipid compound, appeared in the cornea following injury. The presence of PAF triggers various biochemical reactions linked to inflammation and the healing process. PAF quickly activates phospholipase A2, leading to the production of arachidonic acid and specific production of prostaglandins in the corneal epithelium. The infiltration of inflammatory cells invading the amniotic membrane and corneal stroma may be attributed to the effects of platelet activating factor. Large number of eosinophils were observed infiltrating the entire thickness of the cornea. Gris *et al* (2002) in their study noted a thin band of eosinophilic infiltrate over the central cornea corresponding to the amniotic membrane graft. The predominant eosinophils in the initial days of observation may be due to the sutured graft material in the current study. Epithelial hyperplasia and fibroblasts were observed at the edges of the grafted corneal defect and could be considered as proof of wound healing and reepithelialisation being initiated from the edges of the wound in the induced corneal defect as reported by Pepose and Ubels (1992) who stated that corneal epithelial defects heal through a process involving epithelial sliding and mitosis. After a brief lag period of about one hour, the normal epithelium at the edge of the defect flattens, retracts, thickens, and loses its hemidesmosomal attachments to the basement membrane. The epithelial hyperplasia observed at the margins of the grafted corneal wound could also be due to the sutures applied to fix the amniotic membrane graft to the corneal defect. RBCs and few capillaries were noticed in the corneal stroma subjacent to the created defect along with mild oedema. The corneal vascularity observed in the present study could be attributed pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF) from corneal epithelial cells, endothelium,

stromal keratocytes, immune cells like T-cells, and macrophages following corneal injury as reported by (Philip *et al.* 2000). The authors also reported that these vascular endothelial cells secreted enzymes that break down the vascular basement membrane and the surrounding extracellular matrix of the cornea, allowing them to migrate into corneal stroma. The collagen fibres and keratocytes of the corneal stroma subjacent to the created defect were irregularly oriented lacking normal architecture of parallel arrangement which would be the reason for the corneal oedema noticed in the test group in the 7th day of observation.

Histologic examination of group 2 animals on 7th day of observation revealed, corneal defect margins presented with epithelial hyperplasia, and the epithelial cells partially covered the corneal stroma near the edges. According to Jester *et al*. (1999) corneal epithelium heals through migration, mitosis and differentiation of corneal epithelial cells. The epithelial hyperplasia observed at the wound edges in the current study suggests initiation of wound healing. In all the animals of group 2, the corneal stroma revealed infiltrating inflammatory cells like polymorphonuclear cells, lymphocytes, macrophages, and eosinophils. Eosinophilic infiltration along with inflammatory cells noticed in the study could be attributed to the exposure of corneal stroma to the environment. Moderate oedema was noticed. Subjacent stroma presented extensive vascularisation. The collagen fibres and keratocytes of the stroma were irregularly oriented and lost their normal architecture of parallel arrangement. The moderate oedema in the control group may be attributed to the lack of normal architecture of collagen fibrils. More fibroblasts were observed in the stroma. There is epithelial hyperplasia and more number of fibroblasts on the 7th day of observation suggesting the initiation of wound healing.

Histologic examination of group 1 animals on 14th day of observation revealed, absence of demarcation of amniotic membrane from the corneal stroma. The anterior portion of the amniotic membrane was covered by non-keratinising type of stratified squamous epithelium of seven to ten- layer thickness, indicating its hyperplasia. The finding suggests that bovine amniotic membrane derived

extracellular matrix acted as a scaffold and contributed in epithelial healing, by the migration of corneal stroma-derived cells (CSDC) and repopulation into the amniotic stroma as reported by Said *et al*. (2009). The authors also reported that during initial phases of healing, corneal stroma-derived cells extended to the superficial amniotic stroma, while in the later stages, they get repopulated to the deeper layers of the amniotic stroma providing better acceptance of the graft. Plenty of inflammatory cells like polymorphonuclear cells, lymphocytes, macrophages, and fibroblasts were evident in the corneal stroma. Infiltration of inflammatory cells, mainly mononuclear cells and fibroblasts predominated eosinophils. There was a marked reduction in the number of eosinophils. Reduction in the number of inflammatory cells noticed could be attributed to the anti-inflammatory property of bovine amniotic membrane (Tseng *et al.,* 1999, Litwiniuk and Grzela 2014).

Histologic examination of group 2 animals on 14th day of observation revealed corneal defect margins presented epithelial hyperplasia, superficial stratified epithelial cells partially covering the corneal stroma near the edges of the corneal defect alone. The absence of epithelial coverage across the defect suggests that considerable reepithelialization was not happening in the control group. Plenty of inflammatory cells and blood vessels were evident in the corneal stroma. Compared to the group 1 animals there was no reduction in the number of inflammatory cells. Marked oedema was noticed. The number of fibroblasts observed in the stroma was less than that of 7th day observation. The number of fibroblasts observed in the stroma was less when compared to that of 7th day of observation suggesting that considerable wound healing was not happening in the control group. The collagen fibres and keratocyte cells of the stroma were irregularly oriented, and thus, the stroma hence appeared disorganised in histology sections. Corneal epithelial wounds heal through coordinated actions such as cell migration, proliferation, adhesion, and differentiation resulting in the stratification of cell layers. This process relies on interactions mediated by growth factors, cytokines, and signals from the extracellular matrix at the wound site. These interactions aid in regaining epithelial integrity and normal function, as reported by Ljubimov and Saghizadeh, (2015). There was no cell migration, proliferation,

adhesion, and differentiation resulting in the stratification of cell layers in the group 2 which is evident from the histological examination on 14th day of observation.

Histologic examination of group 1 animals on 28th day of observation revealed anterior portion of the amniotic membrane was covered by non keratinised stratified squamous epithelium seven to eight layer thickness. Complete reepithelialisation was observed in the animals on 28th day of observation and correlated with the absence of fluorescein dye staining. Inflammatory cells like polymorphonuclear cells, lymphocytes, macrophages, and fibroblasts were evident in the corneal stroma. However, their numbers were considerably less than that 14th-day observation. Blood vessels and capillaries were evident at the corneal wound edges. The fibroblasts were more elongated and arranged parallel to the epithelium, indicating its transition to fibrocytes and restoration of corneal collagen and normal corneal stromal architecture. Oedema was considerably less than that of the 14th-day observation. The reduction in oedema and regaining of corneal clarity in group 1 animals could be attributed to the restoration of corneal collagen arrangement regaining its normal architecture of parallel arrangement. Newly formed extracellular matrix and more collagen fibres were evident in the stroma of the wound area. Gris *et al.* (2002) also opined that in stromal healing, amniotic membrane initially fills the defect and later get substituted by fibroblasts and collagen which was in accordance with the findings in the current study.

Histologic examination of group 2 animals on 28th day of observation revealed a thick, stratified, hyperplastic epithelium covering the anterior part of the corneal defect at the wound margins alone with no epithelial migration across the defect. The stroma immediately subjacent to the epithelium was disorganised as the enclosed collagen fibres, and fibroblasts were irregularly oriented. According to Schultz *et al*., (1992) stromal replacement involves collagen synthesis and cross-linking, proteoglycan production, and gradual wound remodelling. In animals of group 2 there was no evidence of stromal remodelling and reepithelialisation. The oedema noticed was less than that in the 14th day of observation. There were fewer fibroblasts, inflammatory cells, and blood vessels compared to that of 14th day

observation. As per Schultz *et al*., (1992) keratocytes transform into fibroblasts, which proliferate and rapidly synthesize collagen and other extracellular matrix (ECM). The lower number of fibroblasts observed in group 2 animals indicates a reduced collagen synthesis and extracellular matrix formation compared to group 1 animals.

6. SUMMARY

Bovine amniotic membrane (BAM) derived extracellular matrix (ECM) scaffold was evaluated for healing of corneal wounds in 18 adult healthy rabbits procured from the Small Animal Breeding Station, Mannuthy. Bovine amniotic membrane was collected aseptically during caesarean section. The amnio-chorion thus collected was cleaned off blood clots by washing with normal saline. The amnion was then peeled from the chorion. The obtained amniotic membrane was then washed in phosphate-buffered saline (PBS) containing a mixture of penicillin (50µg/mL), streptomycin (50µg/mL) and amphotericin B (2.5µg/mL). The amniotic membrane was then washed in cold PBS.

The bovine amniotic membrane (BAM) was decellularized by freezing cycle in liquid nitrogen (-196^{0}C) for 22 hours followed by unfreezing in a serological bath at 37^{0}C for two hours. The membrane was then immersed in Tween 80 for four hours. BAM were rinsed with distilled water to remove the remnants of the reagent. After each decellularization step, this rinsing procedure was repeated to remove remnants of the reagents. BAMs were then immersed in 0.1 M NaOH for one hour, later in 0.1 M ascorbic acid for 12 hours. The membrane thus obtained was dipped in 70 % ethanol for one hour to remove residual nucleic acids and phospholipids and finally washed with PBS for two hours. During the entire procedure, membrane was mechanically stirred in an orbital shaker to ensure a homogenous wash. The final sample was subjected to histological studies to rule out cellularity. The decellularised membrane thus obtained was preserved at -20°C till further use.

Animals studied were subjected to detailed ophthalmologic evaluation for presence and nature of ocular discharge, visual function, nature and number of blinks, conjunctival changes, corneal clarity, corneal oedema and vascularisation, fluoresceine dye retention, and tear production. A six millimetre central corneal defect was created in both the test (Group 1; n=9) as well as in the control (Group 2; n = 9) group with a corneal trephine. The central corneal defects of the test group

received decellularized bovine amniotic membrane as an inlay graft sutured to the wound edges with 10-0 nylon. A third eyelid flap was kept in place for 3 days. Topical antibiotics were administered for seven days and oral anti-inflammatory for three days. Elizabethan collar was provided to prevent self mutilation. Control group received topical ocular antibiotic and oral anti-inflammatory alone. Corneal healing was assessed in both test and control groups on 7th, 14th and 28th post-operative days. Culture and antibiotic sensitivity studies of corneal swabs were carried out on 7th, 14th and 28th days of observation. Three animals from each group were sacrificed and corneas were collected on 7th, 14^{th,} and 28th postoperative days and healing was observed histologically.

Abnormal ocular discharge was noticed in both test and control groups on 7th and 14th days of observation, which subsided on 28th day of observation. Blinks were not noticed within a minute in both groups. Dazzle reflex was positive and pupillary light reflex was sluggish. Conjunctival congestion was noticed in the test group, while it was less in the control group. Considerable reduction in oedema and improvement in clarity was noticed in the central cornea in the test group on 28th day of observation. Visible vascularisation was noticed in test group on all days of observation, while it was absent in the control group. STT1 was significantly lower in the control group when compared to the test group. Fluorescein dye staining was negative in the test group on 28th day of observation, while it was positive in the control group. Culture and sensitivity of corneal swabs from both groups revealed gram-positive cocci which was sensitive to amikacin and gentamicin while resistant to moxifloxacin, ofloxacin, and ciprofloxacin.

Histology of cornea in the test group revealed epithelial invading from the wound margins and continuous with the anterior margin of the amniotic membrane on 7th day of observation. Inflammatory cells, fibroblasts, and eosinophils were found abundant in the corneal stroma and amniotic membrane on 7th and 14th day of observation which was found considerably reduced on 28th day. Presence of red blood cells and capillaries were evident on all days of observation along with oedema. On 7th and 14th day of observation, the stroma subjacent to the created

corneal defect had irregularly oriented collagen fibres and keratocytes, lacking normal architecture of parallel arrangement. Restoration of the extracellular matrix and normal collagen was noticed on 28th day of observation. Complete reepithelialisation of the corneal defect was also noticed on 28th day.

Histology of cornea in the control group on 7th and 14th day revealed epithelium invading from the wound margins but not covering the stroma of the corneal defect. On 7th day of observation. Inflammatory cells, fibroblasts, and eosinophils were found abundant in the corneal stroma, which was found considerably reduced on 28th day. Presence of red blood cells and capillaries along with oedema was noticed on all days of observation. On all days of observation, the stroma subjacent to the created corneal defect had irregularly oriented collagen fibres and keratocytes, lacking normal architecture of parallel arrangement. Reepithelialisation was found limited to the wound edges alone in the control animals on all days of observation. Complete reepithelialisation was not observed in the control animals.

From the observations in the current study, it could be thus concluded that

1. Bovine amniotic membrane (BAM) can be easily harvested during caesarean section and decellularized effectively by the chemical method adopted in this study.
2. The BAM derived extracellular matrix (ECM) scaffold could be stored at -20°C in phosphate buffered saline till further use.
3. The BAM derived ECM scaffold helped in differentiation of fibroblast to fibrocytes and reorganisation of the corneal collagen matrix to its normal parallel architecture, and thereby reduced the central corneal oedema and haziness due to the induced corneal injury.
4. The BAM derived ECM scaffold has epitheliotropic effects which helped in complete reepithelialisation of the corneal defect evident histologically as well as negative fluorescein dye uptake.

5. The BAM derived ECM scaffold effectively healed corneal wounds by epitheliotropic effects and reorganising the corneal collagen matrix to its normal parallel architecture in rabbit model.

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PROFORMA FOR CLINICAL STUDY

DEPARTMENT OF VETERINARY SURGERY AND RADIOLOGY,

COLLEGE OF VETERINARY &ANIMAL SCIENCES, POOKODE

Ophthalmologic examination:

PARAMETERS	OD		OS	
Ocular discharge (quantity)	☐ Severe ☐ Moderate ☐ Mild ☐ Absent		☐ Severe ☐ Moderate ☐ Mild ☐ Absent	
Ocular discharge (type)	☐ Serous ☐ Mucoid ☐ Seromucoid ☐ Mucopurulant		☐ Serous ☐ Mucoid ☐ Seromucoid ☐ Mucopurulant	
Conjunctival Changes	☐ Generalised Congestion ☐ Injection of vessels ☐ Severe ☐ Moderate ☐ Mild ☐ Absent		☐ Generalised Congestion ☐ Injection of vessels ☐ Severe ☐ Moderate ☐ Mild ☐ Absent	
Pupillary light reflex	☐Present ☐ Absent		☐ Present ☐ Absent	
Dazzle Reflex	☐Present ☐ Absent		☐ Present ☐ Absent	
Nature & No. of Blinks per minute	Complete	Incomplete	Complete	Incomplete
STT I	………. mm/min		………. mm/min	
STT II	………. mm/min		………. mm/min	
FDT	☐ Positive ☐ Negative		☐ Positive ☐ Negative	

Direct Ophthalmoscopy		
Corneal Clarity	□ Clear (4+) □ Hazy (3+) □ Moderate Opacity (2+) □ Complete Opacity (1+)	□ Clear (4+) □ Hazy (3+) □ Moderate Opacity (2+) □ Complete Opacity (1+)
Corneal Edema	□ No Edema (0) □ Mild (1) □ Marked (2) □ Severe (3)	□ No Edema (0) □ Mild (1) □ Marked (2) □ Severe (3)
Corneal neovascularisation & Score	□ No visible Vessels (0) □ Mild superficial vessels (1) □ Profuse superficial Vessels (2) □ Extensive with vessels from all quadrants (3)	□ No visible Vessels (0) □ Mild superficial vessels (1) □ Profuse superficial Vessels (2) □ Extensive with vessels from all quadrants (3)
Corneal Swab for culture & ABST		

8. ABSTRACT

The study was conducted in 18 adult healthy rabbits procured from the Small Animal Breeding Station, Mannuthy. They were randomly divided into two groups- group 1 and 2 of 9 animals each. Group 1 acted as the test group while the other acted as control group. Bovine amniotic membranes were collected aseptically during caesarean sections. The amnio-chorion thus collected was cleaned off blood clots by washing with normal saline. The amnion was then peeled from the chorion. The obtained amniotic membrane was then washed in phosphate-buffered saline (PBS) containing a mixture of Penicillin (50µg/mL), Streptomycin (50µg/mL) and Amphotericin B (2.5µg/mL). The amniotic membrane was then washed in cold PBS. The membrane was decellularised and preserved at -20°c till further use. A six millimetre central corneal defect was created in both the groups using a corneal trephine. The test group received decellularised bovine amniotic membrane as an inlay graft sutured to the wound edges with 10-0 nylon protected by a third eyelid flap which was retained for three days. Control received topical antibiotic eye drops. Corneal healing was assessed in both test and control group on 7th, 14th and 28th day post-operatively.

Abnormal ocular discharge was noticed in both test and control groups on 7th and 14th days of observation, which subsided on 28th day of observation. Blinks were not noticed within a minute in both groups. Dazzle reflex was positive and pupillary light reflex was sluggish. Conjunctival congestion was noticed in the test group, while it was less in the control group. Considerable reduction in oedema and improvement in clarity was noticed in the central cornea in the test group on 28th day of observation. Visible vascularisation was noticed in test group on all days of observation, while it was absent in the control group. STT1 was significantly lower in the control group when compared to the test group. Fluorescein dye staining was negative in the test group on 28th day of observation, while it was positive in the control group. Culture and sensitivity of corneal swabs from both groups revealed

gram-positive cocci which was sensitive to Amikacin and Gentamicin while resistant to Moxifloxacin, Ofloxacin, and Ciprofloxacin.

Histopathology of the cornea in the test group following amniotic membrane grafting revealed reepithelialisation of two to three layer thickness on 7th day of observation. Epithelial thickness was found seven to ten layer on 14th and 28th day of observation. Reepithelialisation was found complete across the defect on all days of observation in the test group. The collagen fibres and keratocytes of the stroma were irregularly oriented and lost their normal architecture of parallel arrangement in the test group on 7th and 14th day of observation while the fibroblasts were more elongated and arranged parallel to the epithelium, indicating its transition to fibrocytes and restoration of the corneal structure on 28th day of observation.

Histology of the cornea in the control group revealed epithelial invasion from wound edges on 7th day of observation along with fibroblasts. On 14th and $28^{th,}$ day of observation epithelialisation was found to be limited to the wound edges and was lacking across the defect. The collagen fibres and keratocytes of the stroma subjacent to the created defect were irregularly oriented and without normal architecture of parallel arrangement on all days of observation. There were less number of fibroblasts towards the 28th day of observation. Lack of reepithelialisation across the defect, irregularly oriented stromal collagen matrix and less number of fibroblasts indicated improper corneal healing in the control group.

From the study it could be thus concluded that the BAM derived ECM scaffold has epitheliotropic effects which helped in complete reepithelialisation of the corneal defect, and also helped in differentiation of fibroblast to fibrocytes and reorganisation of the corneal collagen matrix to its normal parallel architecture.

Acad. Form No. 19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY
Faculty of Veterinary and Animal Sciences
PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS DEGREE

1. Title of thesis

Bovine amniotic membrane derived extracellular matrix scaffold for corneal wound healing in rabbit model

2. a. Title of the departmental/KVASU research project of which this forms apart

Nil

b. Code No. if any, and order by which departmental/KVASU research projectis approved

Nil

3. a. Name of student

Dr. Krishnakanth K.

b. Admission No.

21-MVP-21

c. Name of the discipline

Veterinary Surgery and Radiology

4. a. Name of major advisor

Dr. Sooryadas S.

b. Designation

Associate Professor and Head, Department of Veterinary Surgery and Radiology, College of Veterinary and Animal Sciences, Pookode, Wayanad, 673576.

5. Objective of the study

Study the efficacy of bovine amniotic membrane derived extracellular matrix (ECM) as a scaffold for corneal wound healing in rabbit model

6. Practical/Scientific utility

Prompt and effective repair of large diameter corneal defects is desirable to avoid corneal perforation and restoration of vision. Treatment of corneal lesions is expensive and despite the therapeutic advances over the past few years, these diseases are still difficult to manage, because of lack of donor corneas, less advancement of transplantation surgeries and graft rejection. Transplantation of natural scaffolds, especially amniotic membrane is a viable option for treating corneal lesions. Decellularisation removes cells and nuclei from the graft mat

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making it less immunogenic. Amniotic membrane gains its popularity as a surgical scaffold because it provides a base for structural reestablishment and contains several bioactive molecules as growth factors, cytokines, and protease inhibitors. These bioactive factors influence cell proliferation as well as anti-inflammation, anti-angiogenesis, anti-scarring, and regeneration properties. These properties play a pivotal role in repairing the ocular surface.

Decellularised bovine amniotic membrane will be blissful to the patients because of its availability and wound healing properties. Hence, preclinical trials and histological studies in lab animal models will help in standardising bovine amniotic membrane derived extracellular matrix as scaffolds for corneal wound healing.

7. Important publications on which the study is based

Ahearne and Lynch (2013) reported that decellularisation preserved the natural extracellular matrix and reduced the host immune response which caused graftrejection.

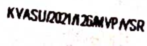
Pontes *et al.* (2014) introduced a surgical technique to treat corneal perforations in experimental rabbits using bovine amniotic membrane and tissue adhesive. The authors concluded that bovine amniotic membrane initiated and accelerated tissue repair, protected cornea, and restricted infiltration of polymorphonuclear cells.

Stepp *et al.* (2014) opined that typical corneal wound size used in rabbits is 6 mm and such circular wounds could be induced using a corneal trephine or a rotating burr of suitable size.

Gopinathan *et al.* (2017) reported that subconjunctival injection of rabbit bone marrow mesenchymal stem cells healed lamellar keratectomy defects in rabbits successfully compared to caprine amniotic membrane.

Costa *et al.* (2019) described the surgical technique and outcome of amniotic membrane transplantation for treatment of complicated corneal ulcers in dogs. The authors concluded that amnioticmembrane transplantation had highly satisfactory visual and cosmetic outcomeswith minimal complications in dogs.

Ballesteros *et al.* (2020) indicated that



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tween 80, sodium hydroxide 0.1 *M*, per acetic acid + ascorbic acid 0.1 percent, ethanol 70 percent, and phosphate buffered saline efficiently removed the cellular contents and preserved the native ECM architecture.

Nagashree *et al.* (2020) after a study conducted in six dogs with corneal ulcers reported that decellularised processed human amniotic membrane as onlay graft was useful in healing of superficial to deepand extensive corneal ulcers in dogs.

John *et al.* (2023) reported that fresh preserved canine amniotic membrane successfully healed corneal wounds in dogs.

8. Outline of technical programme

The study will be conducted at the department of Veterinary Surgery and Radiology, College of Veterinary and Animal Sciences, Pookode. Bovine amniotic membrane will be harvested during cesarean section, and decellularised according to Ballesteros *et al.* (2019) to prepare the ECM scaffold.. The ECM scaffold thus prepared will be stored at -20 ^{0}C till further use.The study will be conducted on 18 apparently healthy adult rabbits procured from CCSEA (Committee for Control and Supervision of Experimentation on Animals) approved breeding stations. Corneal wounds of diameter 6 mm will be induced as per Stepp *et al.* (2014) in the right eye of rabbits. The rabbis will be divided into two groups with 9 animals each. Group 1 will act as the test group, while the other will act as the control. Corneal wounds in the group 1 will be grafted with bovine amniotic membrane-derived ECM scaffold. The grafted scaffold will be protected by a third eyelid flap. Topical antibiotic eye drops and oral anti-inflammatory drugs will be administered post-operatively. Group 2 animals will receive topical antibiotic eye drops and oral anti-inflammatory drugs alone. Necessary precautions to prevent self-mutilation will be adopted as required Throughout the period of study animals will be maintained as per CCSEA guidelines.

Ophthalmic examination will be done on days 7, 14 and 28 post-surgery, to assess healing of the cornea. Corneal swabs will be collected and subjected to antibiotic sensitivity test (ABST) on days 7, 14, and 28 post-surgery. Following ophthalmologic examination on the observation days, three animals each from each group will be sacrificed in humane manner and cornea will be harvested for histologic studies. The data

will be recorded and statistically analysed using appropriate tools. The carcass will be disposed as per CCSEA guidelines.

9. Main items of observation to be made

1. Presence and nature of ocular discharge
2. Results of visual function tests
   a. Pupillary light reflex
   b. Dazzle reflex
3. Nature and number of blinks per minute
4. Conjunctival changes
5. Corneal clarity
6. Corneal oedema and vascularisation
7. Results of Schirmer tear test (mm/ min.)
8. Results of Fluorescein dye test
9. Results of ophthalmoscopic examination
10. Results of culture and antibiotic sensitivity test of corneal swab
11. Histologic observations during healing process

10. Facilities

a. Existing

Existing facilities at the department of Veterinary Surgery and Radiology and other departments of the College of Veterinary and Animal Sciences, Pookode will be utilised.

b. Additional

Microsurgical instruments, chemicals, culture media, suture materials, experimental animals, cages,feed

11. Duration of study

Four semesters

12. Financial estimate

a. Preparation of bovine Amniotic membrane microsurgical instruments, lab animals, suturematerials, feed : Rs20000

b. Miscellaneous : Rs 5000

c. Total : Rs 25000

Signature of student

Project co-ordination group:

Infection Biology

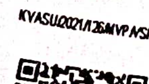
Signature of major advisor

Place: Pookode

Date: 21.11.2023

Name, address and signature of other members of the Advisory Committee

1. Dr. S. Anoop
   Professor,



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Pookode – 673 576.

13. References

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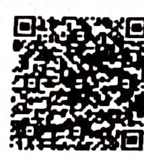

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Pontes, K.C.D.S., Borges, A.P.B., Eleotrio, R.B., Frazao, A.C.D.N., Machado, D.P.D., Reis, E.C.C., Sepulveda, R.V., Duarte, T.D.S. and Laus, J.L. 2014. A new surgical technique to treat corneal perforations using amniotic membrane and surgical adhesive. *Arq. Bras. Med. Vet. Zootec.* **66**: 655-664.

Stepp, M.A., Zieske, J.D., Trinkaus-Randall,V., Kyne, B.M., Pal-Ghosh, S., Tadvalkar, G. and Pajoohesh-Ganji, A. 2014. Wounding the cornea to learn how it heals. *Exp. Eye Res.* **121**:178-193.

14. Time frame of work

Semester III

1. Collection of literature
2. Preparation and submission of research proposal

Semester IV

1. Collection of literature
2. Procurement of materials required forstudy
3. Getting necessary approvals

Semester V

1. Animal experimentation

Semester VI

1. Completion of research work
2. Compilation of data and statistical analysis
3. Preparation and submission of thesis

CERTIFICATE

Certified that the research project has been formulated observing the stipulation laid down under the Prevention of Cruelty to Animals Act (Amendment, 1998).

Dr. Sooryadas S.
Major advisor

Place: Pookode
Date:21.11.2023

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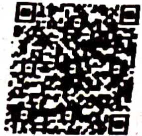

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CURRICULUM VITAE

PERSONAL INFORMATION

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PAPERS/POSTERS PRESENTED

Sooryadas S., Krishnakanth K., Jinesh Kumar N.S., Remya V.., Dinesh P.T., Anju Mary John,, and S. Cibi Mariya. **Canine Amniotic Membrane Grafting for Corneal Surface Reconstruction in a Dog Affected with Bullous Keratopathy.**

Sooryadas S., Krishnakanth K., Jinesh Kumar N.S., Remya V., Dinesh P.T., Sree Lakshmi S.S., Navya P. Shibu, Anu Dinesh and Praveen Kumar M. **Successful Surgical Management Of Corneal Ulcers In Two Dogs Using Meshed Collagen (Colldrez-M) As An Overlay Graft- A Report**

Jinesh Kumar N.S., Krishnakanth K., Sooryadas S., Remya V., Dinesh P.T., Sruthi Chandramohan, and Sree Lakshmi S. S. **Plate-Rod Technique And Cerclage Wiring For The Repair Of Comminuted Diaphyseal Femoral Fracture In A Young Dog**

Jinesh Kumar N.S., Krishnakanth K., Sooryadas S., Remya V., Dinesh P.T., Sruthi Chandramohan, and Sree Lakshmi S. S. **Jejuno-Colic Enteroanastamosis for the Correction of Ileo-Colic Intussusception in a Rottweiler Pup**

Sooryadas S.[2], Krishnakanth K.[1], Jinesh Kumar N.S[3], Remya V.[3], Anu Dinesh[4], Sree Lakshmi S.S[4], Roshna Sadali[4], Navya. P. Shibu[4], Akhilnath R[4], M. Praveen Kumar[4], Saranya M.[4] and Dinesh P.T.[3] **A photographic review of common ocular affections in small animals**

MEMBERSHIP IN PROFESSIONAL SOCIETIES

1. Indian Veterinary Association, Kerala, Pookode
2. Indian Society for Veterinary Surgery