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**RESEARCH ARTICLE**

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

**K. Krishnakanth[1], S. Sooryadas[2]*, V. Remya[3], C.V. Rajani[4], P. Hamza[5] and S. Anoop[6]**

Kerala Veterinary and Animal Sciences University, Pookode, Wayanad- 673 576 (Kerala)

[1]MVSc Scholar, [2]Associate Professor, [6]Assistant Professor, Department of Veterinary Surgery and Radiology; [4]Associate Professor, Department of Veterinary Anatomy; [5]Assistant Professor, Department of Veterinary Pathology, CVAS, Pookode; [3]Professor, Department of Veterinary Surgery and Radiology, CVAS, Mannuthy.

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*In this study, bovine amniotic membrane-derived extracellular matrix was evaluated as a scaffold for corneal wound healing in a rabbit model. The study was conducted in 18 adult healthy rabbits procured from the Small Animal Breeding Station, Mannuthy. Chemically decellularised bovine amniotic membrane (BAM), preserved at -20°c was used for the study. Six-millimetre central corneal defects were created in the right eye of all the rabbits. Test group (n=9) received the decellularized BAM as an inlay graft, while the remaining served as the control receiving topical antibiotic eye drops alone. Corneal healing was assessed in both test and control groups on 7th, 14th, and 28th day postoperatively. Complete healing of the corneal defect, evident from histopathology and absence of fluorescein dye staining, was noticed in the test group on 28th day, while the corneal defect did not heal completely in the control group on 28th day. It could be thus concluded that the BAM derived ECM scaffold has epitheliotropic effects, which helped in complete reepithelialisation of the corneal defect.*

**Keywords**: Bovine amniotic membrane, Corneal wound healing, Extracellular matrix scaffold, Rabbit

Corneal ulcers are serious ophthalmic conditions associated with prolonged healing, pain, discomfort, risk of infection, and reduced quality of life. Accelerating corneal repair and restoring normal function are therefore essential. Among various biological membranes used, amniotic membranes have shown superior healing potential. The amniotic membrane, the innermost fetal membrane, consists of epithelium, basement membrane, and stromal layers that support epithelial cell migration, adhesion, and proliferation. Bovine amniotic membrane (BAM) requires decellularization to reduce immunogenicity and prevent graft rejection. Preclinical evaluation and histological assessment are essential for standardizing BAM-derived extracellular matrix scaffolds for corneal wound healing.

Rabbits are widely used in ophthalmic research due to the size and anatomical similarity of their eyes to those of humans and companion animals. Because of its anti-inflammatory, anti-angiogenic, anti-scarring, anti-fibrotic, and antimicrobial properties, along with its ability to support epithelial regeneration, BAM-derived extracellular matrix was evaluated in this study as a scaffold for corneal wound healing in a rabbit model.

## Materials and Methods

The present study was conducted on 18 healthy adult New Zealand White rabbits of either sex after approval from the Institutional Animal Ethics Committee (IAEC/COVAS/PKD/22/3/2024) at the Department of Veterinary Surgery and Radiology, College of Veterinary and Animal Sciences, Pookode. A 6-mm central corneal defect involving the epithelium and anterior stroma was surgically created in the right eye of each rabbit using a corneal trephine. The animals were divided into two groups: Group I (test group, n=9) received decellularized bovine amniotic membrane (BAM)-derived extracellular matrix (ECM) scaffold as an inlay graft secured with 10-0 nylon sutures, along with topical antibiotics and anti-inflammatory treatment, while group II (control, n=9) received only topical antibiotics and anti-inflammatory therapy.

Clinical evaluation of corneal healing was done on postoperative days 7, 14, and 28. Parameters assessed included presence and nature of ocular discharge, conjunctival congestion, nature and number of blinks per minute, results of visual function tests such as pupillary light reflex, dazzle reflex, ophthalmoscopic examination findings such as corneal clarity, corneal oedema, corneal vascularization, corneal pigmentation, Schirmer tear test I and II, fluorescein dye test, and ophthalmoscopic findings. Corneal clarity was graded from 1+ to 4+, oedema from 0–3, and vascularization from 0–3 based on severity.

Corneal swabs were collected aseptically on days 7, 14, and 28 for bacterial culture and antibiotic sensitivity testing using standard microbiological techniques. Antibiotic sensitivity was evaluated against enrofloxacin, gentamicin, moxifloxacin, tobramycin, ciprofloxacin, ofloxacin, and amikacin according to CLSI (2018) guidelines.

Three animals from each group were euthanised on days 7, 14, and 28 for histopathological evaluation. Corneal tissues were fixed in 10% buffered formalin, processed routinely, stained with haematoxylin and eosin, and examined for corneal oedema, thickening, leukocyte infiltration, neovascularization, and fibroblast proliferation.

Preparation of the BAM-derived ECM scaffold involved aseptic collection of amniochorion from cows undergoing caesarean section. The amnion was

*Corresponding author; E-mail: sooryadas@kvasu.ac.in

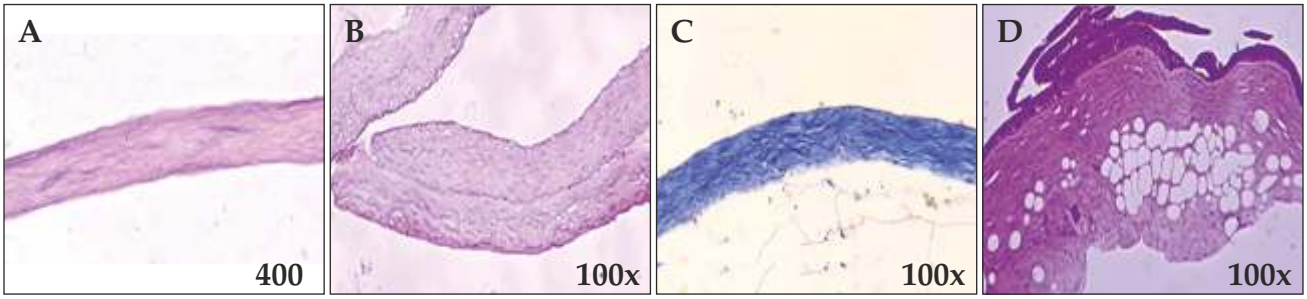



**Fig. 1:** Histology of decellularised BAM (A, B and C) and corneal defect (D): (A) and (B) Decellularised BAM showing collagenous stroma lacking surface epithelium; (C) Gomori's one step trichrome staining showing collagenous stroma of BAM; (D) Histology of removed corneal portion - orange arrow indicates epithelium, black arrow indicates stroma.

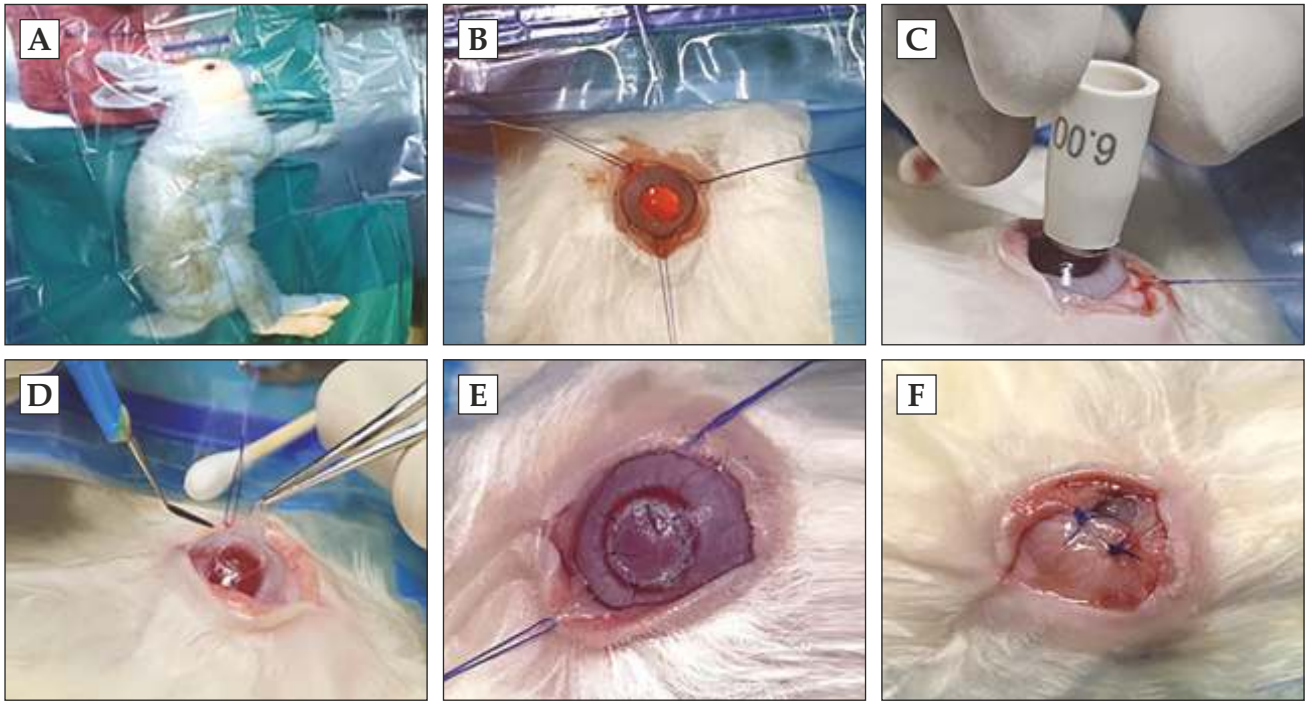



**Fig. 2:** Surgical procedure: (A) Positioning of animal; (B) Fixation of eyeball; (C) Creation of central corneal defect using six millimetre corneal trephine; (D) Removing corneal epithelium and anterior stroma for creating the defect; (E) Amniotic membrane as inlay graft; (F) Third eyelid flap.

peeled from the chorion. The obtained samples were then washed in phosphate-buffered saline (PBS) containing mixture of penicillin (50 µg/mL), streptomycin (50 µg/mL) and amphotericin B (2.5 µg/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 hr followed by unfreezing in a water bath at 37$^0$C for 2 hr. The membrane was then immersed in Tween 80 for 4 hr. 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 1 hr, later in 0.1 M ascorbic acid for 12 hr. The membranes thus obtained were dipped in 70% ethanol for 1 hr to remove residual nucleic acids and phospholipids and finally washed with PBS for 2 hr. 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 (Fig. 1). The decellularised amniotic membrane revealed lack of epithelium and absence of cellularity stroma.

For grafting, the preserved BAM scaffold was trimmed into a 6-mm circular graft and sutured into the corneal defect (Fig. 2) in the test group. A temporary third eyelid flap was maintained for three days postoperatively. All animals received tobramycin eye drops for five days and meloxicam (0.1 mg/kg body weight) for three days, along with Elizabethan collars to prevent self-trauma.

Statistical analysis was performed using independent t-test in SPSS version 24.0, comparing findings between the two groups at each observation period.

**Results and Discussion**

By day 7, group 1 animals exhibited mild mucoid (n=3) or mild serous (n=2) ocular discharge. Group 2 animals showed mild mucoid (n=1), moderate mucoid (n=1), or moderate serous (n=1) discharge. By day 14, discharge was mild serous in group 1 (n=2) and moderate mucoid in group 2 (n=1). Ocular discharge was absent in all other animals at days 7 and 14, and fully resolved across both groups by day 28. Resolution of discharge in group 1 may stem from the antibacterial, anti-inflammatory, and re-epithelializing properties of bovine amniotic membrane (Litwiniuk and Grzela, 2014), facilitating complete clinical and histological corneal healing. Conversely, despite incomplete re-epithelialization, the reduced discharge in group 2 likely reflects the antibacterial efficacy of topical antibiotics.

Across all observation days, pupillary light reflex (PLR) was sluggish in all animals from both groups. This aligns with Warnefors *et al.* (2019), who noted slower, less pronounced direct PLR in rabbits than in dogs. Additionally, the dazzle reflex remained positive throughout, indicating sufficient corneal transparency for retinal light transmission and confirming proper vision. Mechanistically, a positive dazzle reflex denotes an intact retina, optic nerve, optic tract, optic chiasm, supraoptic nuclei, and rostral colliculi (Martin, 2001).

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 10-12 blinks per hour.

On day 7, all group 1 animals showed palpebral conjunctival vessel injection, while group 2 exhibited generalized palpebral conjunctival congestion (n=5) or normal conjunctiva. This hyperemia aligns with Gum *et al*. (1999) regarding corneal epithelial disruption. By day 14, group 1 showed generalized congestion (n=5) and injected vessels (n=1), whereas all group 2 animals were normal. On day 28, group 1 vessel injection (n=1) and generalized congestion (n=2) persisted, while group 2 remained normal. Post-injury conjunctival hyperemia is linked to epithelial proliferation and corneal wound healing (Danjo *et al*., 1987; Shin and Lee, 2006); thus, minimal changes in group 2 likely reflect reduced surgical manipulation of the globe and conjunctiva.

On day 7, group 1 exhibited marked (n=1) or mild (n=1) central corneal oedema, while group 2 showed mild oedema (n=1) with a clear surrounding graft area. This oedema typically arises from stromal water absorption and collagen fiber disarrangement (Moller-Pederson, 2004; Costagliola *et al*., 2013; Samuelson, 2013). By day 14, marked central and peri-graft oedema predominated in group 1, whereas group 2 showed only mild central oedema (n=4). By day 28, mild oedema without peri-graft changes persisted across all animals in both groups. Resolution of oedema in group 1 animals may reflect progressive stromal collagen organization (Lee and Tseng, 1997; Tseng *et al*., 1999), whereas persistent oedema in group 2 likely points to continuous collagen disorientation.

On day 7, mild superficial corneal vascularization seen in group 1 (n=2) and group 2 (n=1). This post-

**Table 1:** Result of comparison of STT1 between group 1 and group 2

| Period | Group 1 (test group) | Group 2 (test group) | T-value | P-value |
|---|---|---|---|---|
| Day 7 | 10.22 ± 0.954 | 10.00 ± 0.527 | 0.024ns | 0.841 |
| Day 14 | 11.50 ± 0.619 | 6.50 ± 0.922 | 4.502** | 0.001 |
| Day 28 | 6.33 ± 1.764 | 7.00 ± 1.732 | 0.270ns | 0.801 |

*** Significant at 0.01 level; ns: non-significant*

**Table 2:** Result of comparison of STT2 between group 1 and group 2.

| Period | Group 1 (test group) | Group 2 (test group) | T-value | P-value |
|---|---|---|---|---|
| Day 7 | 5.33 ± 0.471 | 3.56 ± 0.626 | 2.268* | 0.037 |
| Day 14 | 6.50 ± 0.922 | 2.17 ± 0.477 | 4.174** | 0.002 |
| Day 28 | 7.33 ± 2.333 | 2.67 ± 1.202 | 1.778ns | 0.150 |

*** Significant at 0.01 level; * Significant at 0.05 level; ns: non-significant*

**Table 3:** Results of Fluorescein dye staining in animals of group 1 (test group).

| Animal No. | Day 7 (n=9) | Day 14 (n=6) | Day 28 (n=3) |
|---|---|---|---|
| 1** | + | | |
| 2*** | + | + | |
| 3 | + | + | - |
| 4** | + | | |
| 5*** | + | - | |
| 6** | + | | |
| 7 | + | + | - |
| 8*** | + | + | |
| 9 | + | + | - |

***Animal euthanized on 7th day; ***Animal euthanized on 14th day, for histopathological studies; + staining noticed; - no staining.*

**Table 4:** Results of fluorescein dye staining in animals of group 2 (control group).

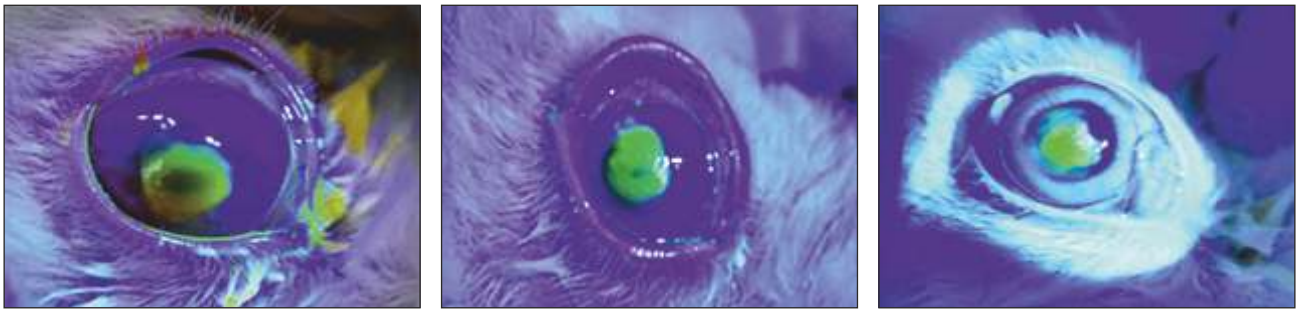
| Animal No. | Day 7 (n=9) | Day 14 (n=6) | Day 28 (n=3) |
|---|---|---|---|
| 1*** | + | + | |
| 2** | + | | |
| 3** | + | | |
| 4*** | + | + | |
| 5** | + | | |
| 6 | + | + | + |
| 7 | + | + | + |
| 8 | + | + | + |
| 9*** | + | + | |

*** Animal euthanized on 7th day; *** Animal euthanized on 14th day, for histopathological studies; + staining noticed.*

Test group (group 1)

**Day 7** **Day 14** **Day 28**

Control group (group 2)



**Day 7** **Day 14** **Day 28**

**Fig. 3:** Results of fluorescein dye staining group 1 (test group) and group 2 (control group) animals at different intervals.

Test group (group 1)

**Day 7** **Day 14** **Day 28**

Control group (group 2)

**Day 7** **Day 14** **Day 28**

**Fig. 4:** Postoperative appearance of cornea in group 1 and group 2 animals at different intervals.

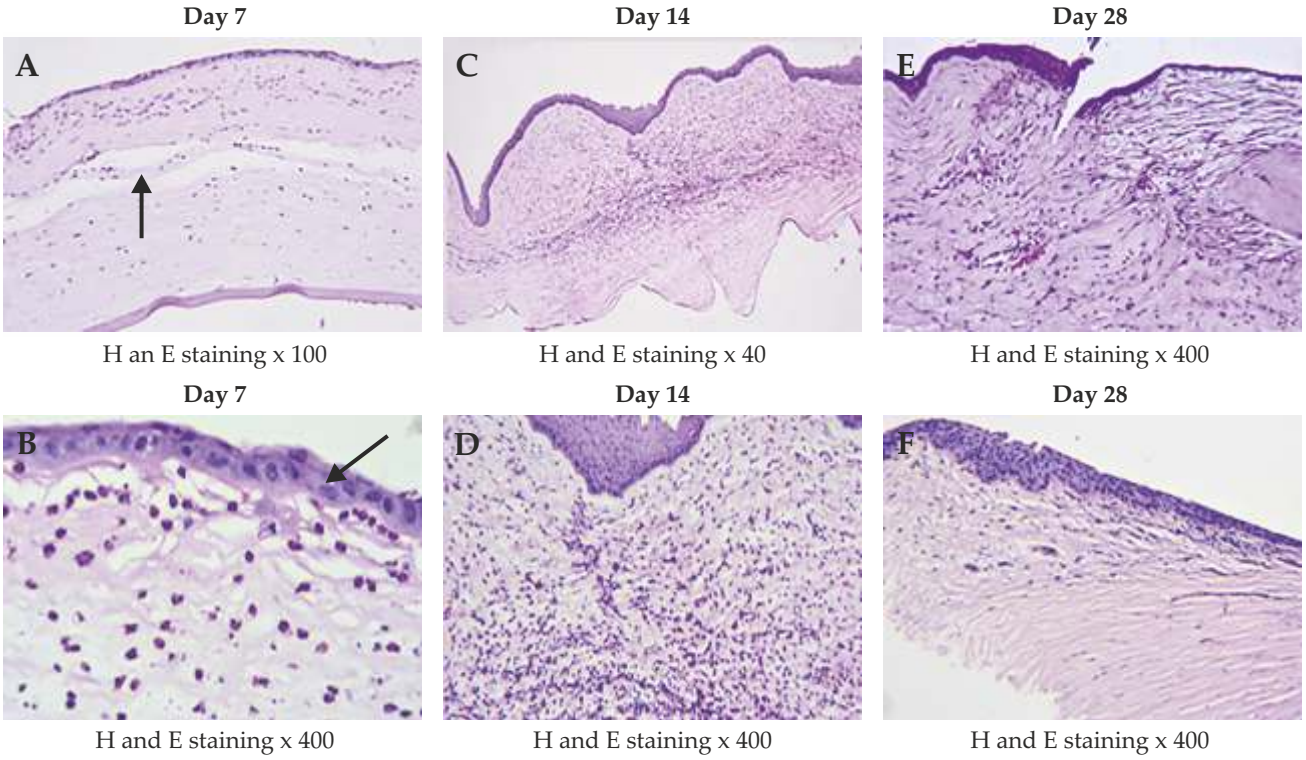



**Fig. 5:** Histological observations in test group 1: (A) Day 7- arrow indicates longitudinal cleft between amniotic membrane and corneal stroma; (B) Day 7- arrow indicate reepithelialisation; (C) Day 14- Arrow indicate reepitheliali-sation; (D) Day 14-infiltration of inflammatory cells; (E) & (F) Day 28- arrows indicate organised collagen arrangement.

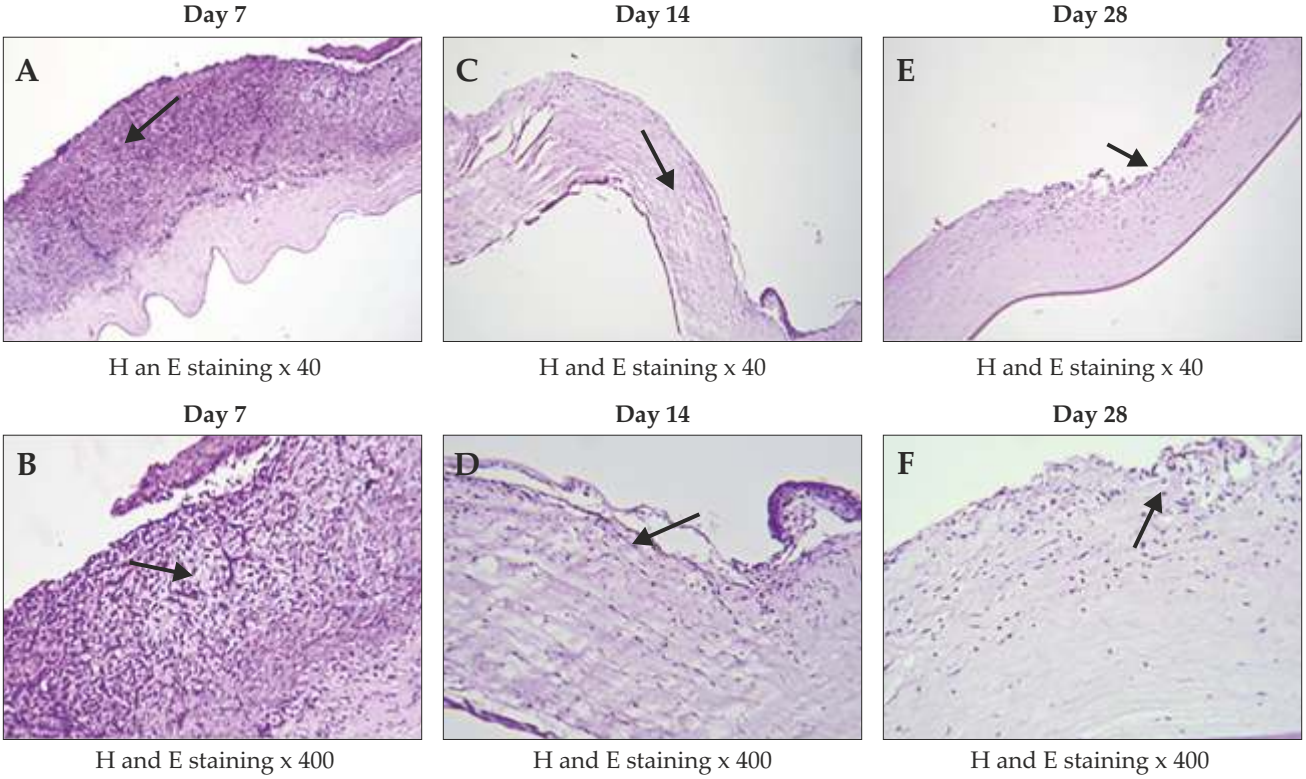



**Fig. 6:** Histological observations in control group: (A) Day 7- arrow indicates reepithelialisation from wound edges; (B) Day 7- arrow indicates infiltration of inflammatory cells; (C) & (D) Day 14- arrow indicates absence of epithelium and disoriented stromal collagen matrix; (E) & (F) Day 28-Arrow indicates absence of epithelium and disoriented stromal collagen matrix.

injury neovascularization is mediated by pro-angiogenic cytokines like VEGF released from local epithelial, stromal, and inflammatory cells (Philipp *et al.*, 2000; Chang *et al.*, 2001; Burri *et al.*, 2004). By day 14, group 1 exhibited mild (n=3), profuse (n=2), or extensive (n=1) superficial vascularization, while group 2 showed only mild vascularization (n=1). On day 28, mild vascularization persisted in group 1 (n=3) but resolved in group 2. Sustained vascularization in group 1 indicates an active reparative healing response (Brooks *et al.*, 2017). While fresh amniotic membranes are strongly anti-angiogenic, preserved variants primarily exert angiostatic effects by limiting inflammatory and angiogenic mediators (Hao *et al.*, 2000).

Mean STT1 values for both groups are detailed in (Tables 1 and 2). On day 14, group 2 values were significantly lower ($P<0.01$) than group 1, which could be attributed to suture irritation in group 1. Abrams *et al*. (1990) reported a normal rabbit STT value of 5.30±2.96 mm/min. By day 28, values in both groups approached the normal range. This may be attributed to the anti-inflammatory effects of the amniotic membrane in group 1 (Lee and Tseng, 1997; Tseng *et al.*, 1999; Litwiniuk and Grzela, 2014; Capistrano da Silva *et al*., 2021). In group 2, the reduction in STT1 on day 14 may be related to anti-inflammatory and antibiotic therapy, whereas the subsequent increase on day 28 may reflect persistence of the corneal defect. Maggs *et al.* (2017) also attributed lower Schirmer tear test values in rabbits to tear film retention resulting from the absence of superior lacrimal puncta.

Fluorescein staining (Tables 3 and 4) was positive on day 7 across both groups. Staining persisted on day 14 in group 1 (n=5) and all group 2 animals, but by day 28, group 1 became completely negative (Fig. 3) while group 2 remained positive. Because fluorescein selectively retains in the hydrophilic stroma (Wilkie and Whittakkar, 1997), negative results of group 1 indicate complete re-epithelialization facilitated by amniotic membrane scaffolding (Kruse *et al.*, 1999).

Corneal cultures yielded gram-positive cocci sensitive to amikacin and gentamicin but resistant to fluoroquinolones (moxifloxacin, ofloxacin, ciprofloxacin). While gentamicin was effective, tobramycin was preferred due to lower epithelial toxicity, and this fluoroquinolone resistance aligns with rising resistance trends among ocular isolates (Tolar *et al.*, 2006).

On day 7, group 1 histology (Fig. 5) revealed an intact amniotic membrane with initiated 2–3 layered re-epithelialization (Gris *et al.*, 2002). Active healing was evidenced by inflammatory and fibroblastic infiltration, hyperplasia, and neovascularization, the latter likely mediated by injury-induced VEGF release (Philipp *et al.*, 2000). Disorganized stromal collagen and keratocytes contributed to the observed mild oedema.

On day 7, group 2 histology (Fig. 6) showed marginal epithelial hyperplasia and partial defect coverage, indicating early healing via migration and mitosis (Jester *et al.*, 1999). Inflammatory infiltration, moderate oedema, extensive vascularization, increased fibroblasts, and irregular collagen architecture suggested repair initiation despite persistent stromal disorganization.

By day 14, group 1 exhibited complete amniotic membrane integration with 7–10 layers of stratified squamous epithelium, indicating scaffold-mediated stromal cell migration and repopulation (Said *et al.*, 2009). Persistent infiltrates shifted primarily to mononuclear cells and fibroblasts with decreased eosinophils, demonstrating the membrane's anti-inflammatory effects (Tseng *et al.*, 1999; Litwiniuk and Grzela, 2014).

In contrast, group 2 on day 14 exhibited marginal epithelial hyperplasia without substantial migration across the defect. Delayed healing was indicated by persistent inflammation, vascularization, marked oedema, reduced fibroblasts, and stromal disorganization, suggesting a lack of coordinated epithelial repair cascades.

By day 28, group 1 corneas achieved complete re-epithelialization (7–8 layers), corresponding with negative fluorescein staining. Minimal oedema, reduced inflammation, and parallel, elongated fibroblasts signaled fibrocyte transition and stromal architectural restoration. This supports observations that the amniotic membrane is gradually replaced by fibroblasts and collagen during healing (Gris *et al.*, 2002).

On day 28, epithelium of group 2 animals remained confined to wound margins with a disorganized subjacent stroma. Despite reduced oedema, inflammation, and vascularization compared to day 14, remodelling and re-epithelialization were minimal. Because stromal repair depends on fibroblast-driven matrix synthesis (Schultz *et al.*, 1992), this diminished fibroblast population indicates impaired collagen deposition and healing relative to group 1.

**Conclusions**

From the observations in the current study, it is concluded that bovine amniotic membrane (BAM) can be easily harvested during caesarean section and decellularized effectively by the chemical method adopted in this study. The BAM derived extracellular matrix (ECM) scaffold could be stored at -20°C in phosphate buffered saline till further use. The BAM derived ECM scaffold helped in differentiation of fibroblasts 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. 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.

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