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Experimental evaluation of safety and efficacy of plasma-treated poly-ε-caprolactone membrane as a substitute for human amniotic membrane in treating corneal epithelial defects in rabbit eyes

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Purpose: To evaluate biocompatibility and safety of plasma-treated poly-ε-caprolactone (pPCL) membrane compared to the human amniotic membrane in the healing of corneal epithelial defects in an experimental model. **Methods:** This is a prospective, randomized animal study including 12 rabbits. Circular epithelial injury measuring 6 mm in diameter was induced over the central cornea of one eye in twelve rabbits. The rabbits were randomized into two groups; in group A, the defect was covered with human amniotic membrane, while in group B, an artificial membrane made of bio-polymer plasma-treated poly-ε-caprolactone was grafted. Six rabbits were euthanized after 1 month and the other six after 3 months and the corneal epithelium was evaluated histopathologically and with immunohistochemistry. **Results:** Light microscopy of the corneal tissue performed after 1 month and 3 months demonstrated similar findings with no significant complications in either group. Immunohistochemistry with anti-CK-3 antibody showed characteristic corneal phenotype in the healed epithelium. In eyes grafted with pPCL membrane, epithelial healing as estimated by a decrease in size of the defect was significantly better than the group treated with the human amniotic membrane at all time periods monitored ($P < 0.05$), except day 1 ($P = 0.83$). The percentage reduction in the size of the epithelial defect was also significantly more in the pPCL membrane group as compared to the human amniotic membrane at all time periods ($P < 0.05$ at all observations) post-implantation except day 1 ($P = 0.73$). **Conclusion:** Plasma-treated poly-ε-caprolactone membrane is safe, biocompatible, and effective in the healing of corneal epithelial defects in rabbits.

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A healthy corneal epithelium is essential for maintaining the transparency and avascularity of the cornea and any severe damage to its integrity can lead to potentially blinding complications. Corneal epithelium as such has good regenerative capability and involves interactions between the epithelial cells and stromal extracellular matrix along with proliferation and migration of epithelial cells.[1,2] Various growth factors and cytokines like transforming growth factor- β (TGF-β) and basic fibroblast growth factor (bFGF) modulate these interactions.[1,2] Presence of risk factors such as dry eye, limbal stem cell deficiency (LSCD), chemical/mechanical trauma, medications, infections, corneal surgery, and systemic diseases can compromise the regenerative ability of corneal epithelium and result in non-healing epithelial defects.[3,4] If not adequately treated, these non-healing epithelial defects can cause significant visual morbidity secondary to opportunistic infections, stromal ulceration, melt, and corneal opacity.

Human amniotic membrane (HAM) transplant is widely used for promoting epithelial healing in situations wherein the epithelial healing is impaired, i.e., acute chemical injury, limbal stem cell deficiency, and neurotrophic ulcers with persistent epithelial defects. However, HAM being an allogenic biological material is associated with certain disadvantages including the potential risk of disease transmission, limited tissue availability and shelf life, biologic variability between tissues, need for specific storage conditions, and economic burden.[5,6] Therefore, the use of a cheaper non-biologic substrate that can help overcome these limitations is much needed and many synthetic substrates like collagen scaffolds, poly (lactide-co-glycolide), polymethacrylate, poly (ethylene glycol), hydroxyethyl-methacrylate, and poly-ε-caprolactone (PCL) are continuously being explored for this purpose.[7]

Among these materials, PCL is gaining much popularity primarily due to its biodegradable aliphatic ester, established drug-delivery models with approval from the USA-Food and Drug Administration, and its use as a bio-engineering scaffold

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or bone graft substitute. PCL has already been studied as drug delivery agents for ocular use and as a carrier to cultivate retinal and conjunctival progenitor cells.[8-10]

We have previously reported that nanofibrous PCL was successfully used as an effective scaffold for the *ex vivo* culture of human corneal epithelial cell line and limbal epithelial cells and demonstrated that the human corneal epithelial cell line expanded on the PCL films retained a normal corneal phenotype.[11] Limbal epithelial cells grown on PCL films showed similar characteristics compared to those cultured on glass coverslips and HAM.[11] The hydrophilicity of the surface achieved by plasma treatment effectively enhanced the transparency of the substrate and promoted the biocompatibility of plasma-treated poly-ε-caprolactone (pPCL).[12] However, till date, to the best of our knowledge, no study has so far evaluated pPCL for its safety and biocompatibility at the preclinical and clinical levels. In this study, we evaluated the safety profile of pPCL for its application in the rabbit eyes model for healing and repair of corneal epithelial defect induced by chemical injury.

Methods

The study was conducted in accordance with the guidelines of our Institute's Animal Ethics Committee (700/IAEC/12) and ARVO (The Association for Research in Vision and Ophthalmology) Statement for the use of animals in ophthalmic and vision research. This was a prospectively conducted randomized animal study evaluating the safety and efficacy profile of pPCL. Twelve New Zealand white rabbits (weight 2–3 kg) were randomized into 2 groups of 6 eyes each by a random number table.[13,14] In group A, the rabbits were grafted with HAM in one eye, while group B was grafted with a membrane composed of pPCL.

Preparation of HAM, pPCL, and fibrin glue

Cryopreserved HAM, prepared and stored using standard protocol and media, was procured and thawed at room temperature for 10 min before transplantation.[15] PCL pellets were dissolved in trifluoroethanol (TFE) to make a 10% w/v solution of PCL. The solution was electrospun using a dual-polarity high-voltage DC power supply unit (Gamma High Voltage Research, Ormond Beach, FL), a syringe pump (KDS 100; KD Scientific, Holliston, MA), 2 mL syringe, and a 24-G needle with a blunted tip. The positive terminal of the high-voltage supply was connected to the needle tip, while the negative terminal was connected to a metallic collector plate to maintain a voltage of 15 kV between them. The fibers were electrospun at a flow rate of 0.5 mL/h at a tip to collector distance of 13 cm and collected on circular cover-slips kept over the metallic collector plate. After spinning, the coverslips deposited with the PCL nanofibers were removed from the metallic collector followed by plasma treatment in an indigenously designed dielectric barrier discharge atmospheric pressure glow plasma reactor. Helium–oxygen gas mixture (3:1 ratio) was introduced inside the reactor chamber and glow plasma was created at a discharge voltage of 3.5 kV, power 10 W, and frequency of 15 kHz for 2 min to create hydrophilic functional groups on the PCL surface. Tweezers were used to remove the samples. Plasma-treated PCL (pPCL) scaffolds were preconditioned by washing with a phosphate buffer solution containing antibiotics and then irradiated using a UV light for 3 h. The scaffolds were incubated in a culture medium at 37°C overnight prior to experimentation.[11]

Fibrin sealant (Tisseel TM, Baxter International Inc.) was prepared as per the instructions of the manufacturer.

Surgical creation of the epithelial defect

The rabbits were anesthetized using intramuscular injection (quadriceps) of xylazine (35 mg/kg) and ketamine (5 mg/kg).[13,14] A 6 mm × 6 mm circular epithelial defect was created in the center of the cornea of the right eye of each rabbit using a circular filter paper dipped in freshly prepared 1N NaOH under aseptic conditions.[16] The filter paper was momentarily touched to the cornea and removed immediately in both groups to avoid deep injury and scarring. Thorough saline irrigation was done to remove the excess alkali. The size and area of the epithelial defect were noted by staining with 1% fluorescein dye and examining on Micron III imaging system with slit lamp attachment (Phoenix Research laboratories; Pleasanton, CA).

Placement of substrate graft using tissue adhesive

Immediately after the creation of the epithelial defect, the grafts (either HAM or pPCL membrane) were carefully placed inside the defect using fibrin glue. In group A, the membrane was peeled from the nitrocellulose paper and a 6 mm × 6 mm graft was fashioned. HAM was placed with the epithelial side up as a graft over the dried epithelial defect after application of freshly prepared fibrin glue using Duploject system.[17]

In group B, preconditioned pPCL membrane was taken in a sterile container, removed from the surrounding aluminum foil and a 6 mm × 6 mm graft was fashioned from it. Using a similar placement method as HAM, pPCL membrane was placed over the dried epithelial defect after application of freshly prepared fibrin glue using Duploject system.

Post-implantation, rabbit's eyes were covered with a protective shield and topical antibiotic (moxifloxacin 0.5%, 4 times per day), cycloplegic (homatropine 2%, 2 times per day), and lubricants (6 times per day) were administered for 14 days.

Follow-up evaluation

The operated eyes of the rabbits were monitored daily until total healing of the epithelial defects was observed. Six rabbits, three from each group were sacrificed after 1 month and the remaining six rabbits were euthanized after 3 months for histopathological examination of the corneas. Time points of 1 month and 3 months were taken to ascertain the attainment and maintenance of a healthy corneal phenotype by the regenerated epithelium and also to look for any possible evidence of subclinical inflammation. The size of the epithelial defect was measured using 1% fluorescein stain at each examination and the protective eye shield was put back in place. Eyes were examined daily on Micron III imaging system with slit lamp attachment and photographs were captured using Streampix software (Norpix Inc.). A thorough examination was done to look for any possible complications like excessive inflammation, congestion, graft displacement, infective keratitis, scarring, and neovascularization.

Serial measurements of reduction in epithelial defect were done as follows:

i. Epithelial defect area in both groups was measured using ImageJ software [version 1.46r/Java 1.6.0_20 (32-bit), National Institute of Health, Bethesda, USA]. The photographs were analyzed and the epithelial defect was outlined using a polygon after calibrating the scale (Scale = 100 pixels/mm). This area was then calculated by the software.
ii. Percentage reduction in epithelial defect area in both the groups was calculated for each examination using the formula: % reduction = (A0 - AX)*100/A0 where; A0 = Area of epithelial defect on Day 0, AX = Area of epithelial Defect on Day X (X = day for which measurement is required).

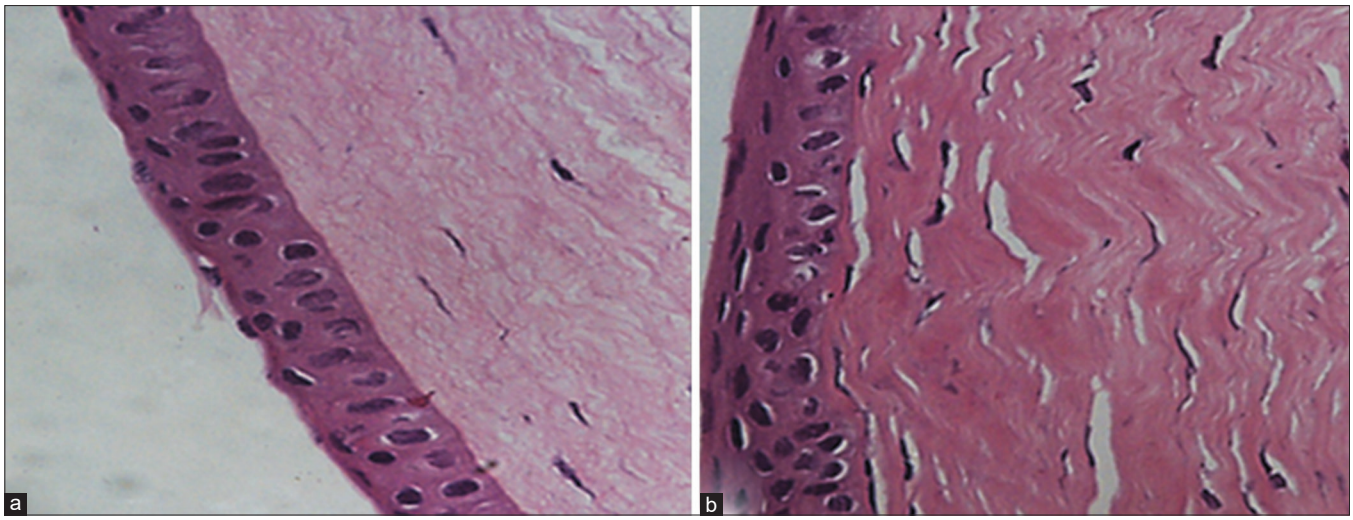

Figure 1: Light microscopic images showing normal epithelium and stroma after healing of epithelial defect both in eyes with human amniotic membrane (a) and in eyes with plasma-treated poly-ε-caprolactone membrane graft (b). There is no evidence of lymphocytic cell infiltration, vascularization, or fibrous tissue (H&E ×200)

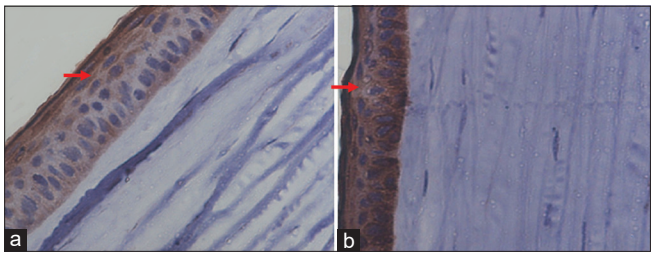

Figure 2: Cytoplasmic CK 3 positivity, as indicated by the chocolate brown staining (red arrows), in the re-epithelialized area can be seen in both the human amniotic membrane group (a) and the plasma-treated poly-ε-caprolactone membrane group (b) and confirms corneal origin of the cells in the re-epithelialized area (Avidin-Biotin ×400)

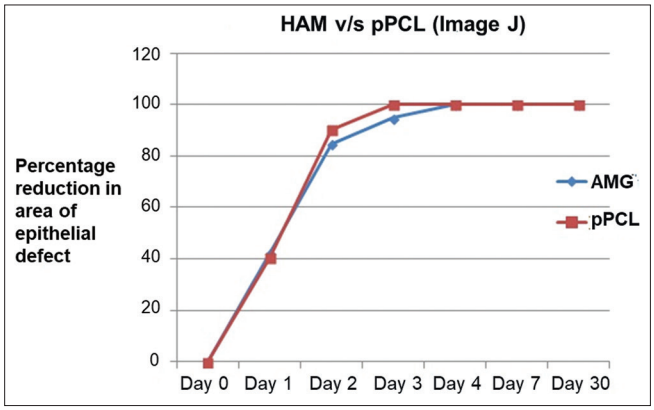

Figure 4: Graphical representation of percentage reduction in the area of epithelial defect in both groups over time

The area used in the formula was measured using ImageJ software as discussed above.

Histopathological examination

Six rabbits (three from each group) were euthanized after 1 month and the remaining six after 3 months and the eyes were processed for histopathology using hematoxylin and eosin (H&E) stain and immunohistochemistry (IHC) using CK-3 antibodies, a differentiated corneal epithelial marker.

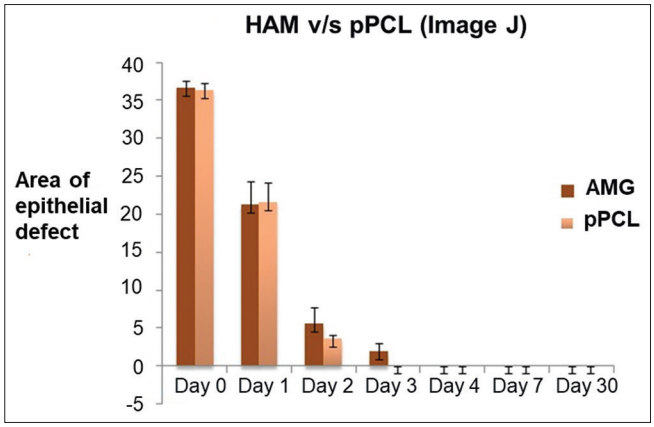

Figure 3: Graphical representation of the area of epithelial defect in both groups over time

Statistical analysis

Data was recorded on predesigned proforma and entered into a Microsoft Excel spreadsheet. The analysis was done using SPSS Statistics v 20.0.0 Software ® (IBM Corp., New York, USA). The data was normally distributed and thus t-test was applied to compare the two groups at each point of time. Repeated measure analysis followed by post hoc comparison by Least Square Deviation (LSD) method was used as a test for change over a period of time. When data was not normally distributed, the Freidman test was applied. A 2-tailed *P* value with $P < 0.05$ was considered statistically significant.

Results

Safety and biocompatibility

Clinical evaluation for complications

At every follow-up evaluation, each of the eyes was carefully examined for any evidence of corneal stromal melt, corneal vascularization, LSCD, conjunctivalization, and/or stromal scarring. Both the groups showed mild-to-moderate

conjunctival congestion, which subsequently subsided within 10 days post-implantation. There was no difference in the extent of congestion between the groups. Both the HAM and pPCL membrane had also disintegrated within the same period of time. At 1 month and 3 months of follow-up, none of the groups showed any signs of complications of ocular chemical injury.

Histopathological evaluation

Light microscopy of the corneal tissue performed after 1 month and 3 months demonstrated no lymphocytic cell infiltration, vascularization, or fibrous tissue in either of the groups. Both groups had similar histopathological features characteristic of re-epithelialized tissue as well as in the surrounding area [Fig. 1a and b]. Immunohistochemistry with anti-CK-3 antibody showed discrete cytoplasmic positivity indicating that the cells of the re-epithelialized area had characteristic corneal phenotype. This was similar in both groups at day 30 and day 90 [Fig. 2a and b].

Efficacy

Reduction of the epithelial defect area

The mean time taken for the epithelial defect to heal was 3.5 ± 0.5 days overall. Measurements of the area of the epithelial defect on day 1 showed comparable sizes between the two groups (21.25 ± 3.01 mm^2 and 21.59 ± 2.56 mm^2 in groups A and B, respectively, $P = 0.83$). On subsequent days, there was a statistically significant difference in the area of the epithelial defect between the 2 groups, with the mean area being lesser in Group B (3.59 ± 0.53 mm^2 and 0 mm^2 on Day 2 and Day 3, respectively) as compared to Group A (5.59 ± 2.09 mm^2 and 1.9 ± 1.08 mm^2 on Days 2 and Day 3, respectively; $P = 0.047$ and 0.008 on Day 2 and Day 3, respectively). The epithelial defect healed completely in all rabbit eyes in Group B by Day 3, while it healed by Day 4 in all eyes in Group A [Fig. 3].

Percentage reduction of the epithelial defect area

There was no significant difference in mean percentage reduction of the epithelial defect area between Group A and Group B on Day 1 (42.09% ± 7.42 vs 40.64% ± 6.47, respectively, $P = 0.73$). The difference was evident on Day 2 (84.74 ± 5.55% vs 90.14 ± 1.36%, respectively, $P = 0.04$) and Day 3 (94.8 ± 2.99 vs 100%, respectively, $P = 0.002$) [Fig. 4].

Discussion

This study was undertaken as one of the first steps in evaluating pPCL for its potential future role as a scaffold for ocular surface epithelial proliferation/healing. The purpose of this pilot study was to find if the pPCL membrane is safe and well-tolerated for ocular use in an animal model and to compare its efficacy to that of HAM. The results indicate that both pPCL membrane and HAM were safe to implant with no indication of any excessive inflammation in the rabbit eyes. However, in terms of their regenerative potential, the pPCL graft was found to be slightly more effective in healing the epithelial defects as compared to HAM within a given environment. Further comparisons by histopathological examinations revealed that the healing process was found to be similar in both the test groups.

HAM is one of the most commonly used substrates for ocular surface reconstruction and tissue engineering of the cornea.[18,19] However, the use of HAM is potentially associated with several risks owing to its biological origin such as disease transmission and immune responses.[20,21] Incidence rates of 1.6%–8.0% have been reported for post-HAM transplantation infection with gram-positive isolates being reported most frequently.[22-24] Some other problems associated with HAM include limited availability, need for a long quarantine period before usage, and need for specific storage conditions which are expensive.[21,25] In contrast, pPCL membrane has been seen to be safe in our study with a similar tissue response observed from HAM. Our previous study has also shown that pPCL has potential for future use in ocular surface reconstruction, limbal stem cell culture, and transplant, and being a synthetic substitute will effectively overcome the above-mentioned limitations offered by HAM for ocular surface reconstruction.[12]

HAM produces several growth factors like (transforming growth factor [TGF], basic fibroblast growth factor [bFGF], hepatocyte growth factor [HGF], and fetal hyaluronic acid), cytokines and proteinase inhibitors. These growth factors help to stimulate epithelialization and differentiation of stromal fibroblasts.[26] HAM also has reported anti-inflammatory action by suppressing the expression of inflammatory cytokines from the ocular surface.[27] Unlike HAM, the pPCL membrane lacks any intrinsic biological property but interestingly, we found that pPCL grafts were almost of similar efficacy in healing the epithelial defects in rabbit corneas despite the absence of supplemented cytokines and growth factors. However, in the future, it may be interesting to investigate the effect of supplementation of pPCL with additional growth factors like autologous serum which may show improved healing responses *in vivo*.

Despite the universal acceptance of HAM, its limitations of biological variability, cost, processing requirements, storage restrictions, perishability, and logistic challenges in availability are also acknowledged as restricting its full potential.[28,29] Some efforts have been made in addressing these remaining concerns over the past few years. Special processing techniques have been applied to permit dry storage at room temperature while retaining the native and regenerative characteristics of the fresh amniotic membrane.[30,31] Even with these advancements, the processing and sterilization of HAM is bound to destroy the fragile biologics to some extent and its efficacy in terms of delivering bioactive cytokines and growth factors is questionable. Notwithstanding the benefits, the human and biological origin of the tissue has inherent disadvantages in terms of potential transmission of prions and other biological substances. In settings where the membrane works purely as a bio-degradable dressing relying on host healing properties, the undoubted benefits of synthetic material are clear particularly the advantages of being sterilized and truly made-to-order. We propose that pPCL can be a useful alternative to amnion with the assurance of sterility, reliability, amenable to quality control, and which can even be supported by a variety of repair inducing constituents by supplementation with autologous serum or a cocktail of proteins, growth factors, and other medications as needed.

One limitation of our study is the inclusion of a small number of rabbits. However, like any other newly introduced biomaterial, such animal studies should be done in a phased manner by gradually increasing the sample size before putting the substance to human use. The epithelial defects created in our study had clean margins and were sterile, and it is assumed that there was no limbal stem cell deficiency. The response of tissue to pPCL in real-time conditions like infections and chemical injury may be different than seen in this study and would need further evaluation. Preparation of pPCL needs a specialized lab and equipment, and this may increase the cost of the membrane compared to HAM. Once pPCL is clinically validated, further cost-effective analysis will need to be done comparing both interventional modalities.

We sought to evaluate an alternative treatment modality for the management of epithelial defects which is easily available off the shelf, free from the risk of disease transmission, has longer storage time, and is cost-effective. The findings of this

preclinical level study establish that pPCL is a safe and effective alternative to HAM for ocular use in iatrogenically induced epithelial defects in rabbit eyes. These findings form the premise for future human clinical trials comparing pPCL to HAM and controls in various disease conditions. In summary, there were no adverse effects observed in both pPCL and HAM implants up to 90 days *in vivo*. Both HAM and pPCL membrane showed similar histopathological and IHC profiles of the healed epithelial tissue, albeit healing was relatively faster in the case of pPCL.

Conclusion

Overall, our study provided concrete evidence that pPCL has a good potential for use as an artificial substrate for ocular surface healing in this initial evaluation in rabbit model and should be analyzed further in appropriately phased studies to reach the level of clinical trial where true benefit can be shown.

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Statement of Ethics

The study was conducted in accordance with the guidelines of the Institute Animal Ethics Committee of the All India Institute of Medical Science, New Delhi, India (700/IAEC/12) and ARVO (The Association for Research in Vision and Ophthalmology) Statement for the use of animals in ophthalmic and vision research.

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Conflicts of interest

There are no conflicts of interest.

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