Engraftment and Proliferation of Thermoreversible-Gelation-Polymer-Encapsulated Human Corneal Limbal-Stem-Cells on Ocular Surface of a Cadaver Cornea

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ABSTRACT

Purpose: Corneal limbal stem cell (LSC) transplantation has been reported as a potential approach to treat the damaged corneal epithelium. Scaffolds such as human amniotic membrane (hAM) are commonly employed for the in vitro culture and as a carrier during in vivo transplantation. However, they carry the risk of biological contamination and donor to donor variability. To overcome these disadvantages, we herein report the capabilities of a synthetic thermoreversible gelation polymer (TGP) scaffold to serve as an encapsulation support during LSC transplantation and to enable engraftment for corneal regeneration.

Methods: Sixteen discarded human corneas were used to isolate the corneal epithelium which was cultured in TGP and hAM. The cell proliferation and characteristics between TGP and hAM culture methods were evaluated by microscopic observation, 3H Thymidine incorporation assay, immunoperoxidase and immunofluorescence staining.

Results: The 3H Thymidine assay’s results showed that TGP allowed human-donor cornea-derived LSCs to proliferate well in vitro, compared to hAM and the cells encapsulated in TGP and transplanted ex vivo onto a human cadaver donor cornea denuded of its epithelium, migrated on the ocular surface, and proliferated to form a continuous layer in 25 days. Immunoperoxidase and Immunofluorescence staining of TGP-cultured cells were positive for LSC markers (p63, ABCG2, Connexin 43 and Integrin b1), proving that the TGP helps to preserve the limbal cells’ stemness.

Conclusion: TGP is found to be a multipurpose scaffold for (i) in vitro culture, (ii) ex vivo encapsulation, and in vivo transplantation (iii), enabling engraftment of LSCs in this study, with potentials to extend its application in cell-based therapies in several regenerative medicine approaches.

Introduction

The cornea is a transparent tissue with numerous cellular and non-cellular layers that is highly organized. The corneal epithelium, which covers the corneal surface, is crucial to transparency and protection.1,2 When the cornea, along with the limbus is damaged due to causes such as chemical injuries, burns or Steven Johnson syndrome, the conjunctiva proliferates over the cornea, causing opacification and vascularization, which in turn impair vision. At the limbus, a tissue rim situated at the intersection of the cornea and sclera, are the stem cells of the cornea called limbal epithelial stem cells (LSCs).2 These LSCs divide asymmetrically to create transit-amplifying cells, which then differentiate to create mature corneal epithelium in the centre of the cornea. Any pathology that directly destroys or damages LSCs is a potential cause of limbal stem cell deficiency (LSCD).2 The mainstay treatment for stage I LSCD is medical care. Alternately, medical management can play an important role in stabilising the progression of the disease in advanced cases involving the central cornea (stages II and III) while the patient awaits definitive surgical treatment.1,3 To optimise the function of the meibomian glands, the initial treatment will consist of prescribing artificial tears, maintaining good lid hygiene, and applying a warm compress.1,4,5 Short- or long-term topical corticosteroid treatment, topical cyclosporine, and antibiotics are also employed. Autologous and allogeneic serum drops are also used. When the resident population of
LSCs is insufficient to replenish the corneal epithelium and the visual axis is affected, surgical treatment is required.\textsuperscript{1} Traditional tissue transplant techniques aim to repopulate the corneal surface with LSCs, which continue to be the most common surgical treatments for LSCD. Surgical procedures for LSCD are classified according to the source of stem cells (e.g. autologous, allogeneic) and type of stem cell graft (e.g. limbal, oral mucosa or hair follicle, among others) as well as the use of \textit{ex vivo} expansion of stem cells in culture. For allogeneic grafts, immunologic rejection continues to be one of the leading causes of graft failure. Penetrating keratoplasty (PKP) is frequently performed as a second procedure after LSC transplantation to replace deeply scarred stroma in order to restore vision.\textsuperscript{1,6} Patients with unilateral LSCD can be transplanted with limbus tissue graft from the healthy contralateral eye. In cases of unilateral LSCD with a healthy contralateral eye, the conjunctival limbal autograft (CLAU) is the most performed procedure. Development of LSCD in the healthy donor eye is a potentially devastating complication.\textsuperscript{1,7–10} For severely damaged ocular surfaces with inadequate conjunctiva and symblepharon, a combined CLAU and living related-conjunctival limbal allograft (lr-CLAL) procedure that maximises transplanted conjunctiva is used. Keratolimbal allograft (KLAL) uses cadaveric peripheral cornea and limbus obtained from eye banks and can thus be used in the case of bilateral LSCD or when the patient with unilateral disease is unwilling to use the healthy other eye as a donor and no living relative or human leukocyte antigen (HLA) match is available. In cases of bilateral LSCD requiring reconstruction of the conjunctiva, the Cincinnati procedure, which is a combination of lr-CLAL and KLAL with systemic immunosuppression, is suggested. Theoretically, the goal of the ideal surgical treatment would be to establish a population of stem cells that can survive over time and renew the corneal epithelium. Ideally, it should also restore a suitable stem cell niche for the survival of these stem cells; however, this goal is probably not met by current therapies.\textsuperscript{1,8–10} This led to the development of Cultivated Limbal Epithelial Cells transplantation (CLET). Unlike CLAU and lr-CLAU, which require a large graft from a healthy eye, CLET only requires a small graft (approximately 2 mm\textsuperscript{2}). Typically, limbal epithelial tissue is expanded as an intact explant; however, biopsies may be digested to produce suspension cultures as well. The stem cell construct is then transplanted onto the ocular surface after approximately two weeks of culture on a carrier scaffold, most frequently human amniotic membrane (hAM) or fibrin.\textsuperscript{1,8–10}

Human amniotic membranes (hAM), fibrin gels, plasma polymer-coated surfaces, biodegradable matrices, and feeder layers of mouse NIH 3T3 cells have been used to culture limbal epithelial cells. hAM, a collagen-based extracellular matrix derived from the human placenta, is widely used as scaffold in cell-based therapies for the cornea. Their disadvantages are heterogeneity of hAM, donor to donor variability, risk of infection and low mechanical strength.\textsuperscript{10,11} Organ decellularization has also been used for CLET, which involves removing cells and their debris to obtain an acellular scaffold composed of only the organ’s extracellular matrix (ECM). By removing the cells, the major histocompatibility complexes are diminished, thereby decreasing the risk of graft rejection. As with many materials, batch-to-batch variation is to be expected due to donor variation. It is also essential that the corneas have been adequately decellularized. Incomplete decellularization has been observed \textit{in vitro} and \textit{in vivo} to result in the polarisation of macrophages toward an M1 phenotype.\textsuperscript{10} In the case of the natural scaffolds described above, they are static microstructures which have immutable pattern geometries and surface properties which may hinder the creation of biomimetic microtissues and the controlled retrieval of tissues from these platforms. In addition, their use necessitates the application of enzymes or physical forces to detach monolayers of tissues, which is undesirable. Hence synthetic polymers are a potential alternative to the above-described materials. These synthetic polymers are dynamic microstructures with controllable features and reversible surface properties.\textsuperscript{12–15} Many synthetic polymers such as PCL and PLLA, have been used as scaffolds to engineer other tissues but have been typically deemed unsuitable for corneal engineering due to their lack of transparency. Polyvinyl alcohol (PVA) is a transparent synthetic polymer which has been combined with natural materials such as cellulose, amniotic membrane, and collagen to generate biocompatible scaffolds for corneal tissue engineering. Poly(N-isopropylacrylamide) (PNIPAAm) is a well-known stimuli-responsive polymer that changes its hydrophilicity and swelling in response to temperature, making it ideal for cell retrieval without the use of enzymes. PEG is another synthetic polymer that has been used in corneal scaffolds. PEG hydrogels have favourable biocompatibility, permeability, and transparency, making them an ideal candidate for corneal replacement.\textsuperscript{12–16}

We have earlier reported a thermoreversible gelation polymer (TGP) (Commercial name: Mebiol gel), which is a synthetic biocompatible copolymer composed of thermo-responsive polymer blocks [poly (N-isopropylacrylamide-co-n-butyl methacrylate) poly(NIPAAm-BMA)] and hydrophilic polymer blocks (polyethylene glycol [PEG]) for the culture of different kinds of cells and stem cells.\textsuperscript{17–26} The advantageous characteristics of TGP include its transparency when it is a hydrogel without syneresis or separation of water and the rapid sol-gel transformation without hysteresis.\textsuperscript{17} The TGP, which is a temperature-dependant dynamic viscoelastic polymer, is transparent, cells/tissues can be added when the TGP is cooled under the lower critical solution temperature (LCST) (the temperature at which the solid and liquid phases separation occurs) and at temperatures above the LCST, for instance at 37°C it becomes a three-dimensional gel-scaffold for the cells/tissues to grow.\textsuperscript{27} To retrieve the cells or tissues from TGP, mere cooling in a refrigerator helps the gel to become sol which can be washed away\textsuperscript{15,18,19} and the cells can be harvested without the use of digestive enzymes. The TGP’s capabilities as a scaffold, have been reported in both \textit{in vitro}\textsuperscript{17,18} animal\textsuperscript{19,20} and human clinical studies,\textsuperscript{23,24} for different kinds of cells (e.g. hepatocytes, buccal mucosal epithelial cells) and stem cells (e.g. bone marrow mononuclear cells, pluripotent stem cells), from both vascular (e.g. bone marrow, liver) and avascular...
primary sources of the tissue (e.g. cartilage). We earlier reported the capabilities of this TGP scaffold in supporting the growth of LSCs, as well as the LSC transplantation in a rabbit model of corneal damage.

We herein investigate the TGP for such characteristics by studying TGP’s capability to allow LSC attachment to a cadaver donor cornea denuded of its epithelium to further understand its potential to serve as an in vivo carrier-scaffold for cell encapsulation and transplantation.

Materials and methods

Informed consent was obtained from the guardians of the deceased donors whose corneas were used to obtain the limbal stem cells. For the use of hAM, informed consent was obtained from the donor undergoing caesarean section. Sixteen corneas which were not found to be usable for transplantation and hence discarded, were used for the study.

TGP and its reconstitution

The culture media was composed of DMEM + Ham’s F-12 Nutrient Mixture (F-12) medium (Gibco, USA) containing 20% fetal calf serum (Sigma Aldrich, St. Louis, MO, USA) along with 1.05 mM calcium, 5 μg/mL crystalline bovine insulin (Sigma Aldrich, St. Louis, MO, USA), 30 ng/mL cholera-toxin (Calbiochem, San Diego, CA, USA), 2 ng/mL epidermal growth factor (EGF, R & D Systems, Inc., Minneapolis, MN, USA), 0.5% dimethyl sulfoxide (DMSO, Sigma Aldrich, USA), 0.5 μg/mL hydrocortisone, 5 ng/mL sodium selenite, and 5 μg/mL apo-transferrin. The same medium was used for the reconstitution of TGP as well as the culture in TGP and hAM.

The TGP was obtained in a lyophilized form from Nichi-In Biosciences Pvt. Ltd., India, through M/s GN Corporation Co. Ltd., Japan. We reconstituted 1g of TGP with 9 mL of the culture medium and placed it in a refrigerator at 4°C overnight, so that it yielded a viscous transparent gel without air bubbles for use in the experiments. A fluorescein-conjugated variant of the TGP was also used to study whether TGP can be completely washed away from the cells to serve as carrier during transplantation. The fluorescent-conjugated TGP was prepared by covalently binding Fluorescein isothiocyanate (FITC) to TGP molecule. The fluorescent-conjugated TGP was purified by using diafiltration with the ultra-filtration membrane with molecular cut off 100,000 Dalton same as that of original TGP. Therefore, the fluorescent conjugates corresponded to the size of TGP compounds. The fluorescein-conjugated variant was also reconstituted with culture medium in a similar manner as described above.

Preparation of hAM

For culture on hAM, the tissue was obtained from a consenting donor after caesarean section. The hAM was separated from the chorion, and then washed free of blood and its clots. The hAM was trypsinized for 45 min and epithelial cells were removed by soft scraping of the membrane using sterile cotton swab. The epithelium-denuded hAM was preserved in DMEM containing glycerol for use as a control base in which corneal limbal tissue was cultured.

Culture of human LSC in TGP and hAM

Each of the sixteen corneas were divided into two portions with one portion being cultured in TGP and the other on hAM. The limbal epithelium was cut from the cornea into 0.5 to 1 mm pieces. For culture in hAM, the hAM was sutured onto a culture plate insert with the basement membrane facing up and placed in a 24-well tissue culture plastic plate with nonabsorbable sutures. The limbal tissue pieces were added onto the denuded amniotic membrane. Once the tissues adhered to the hAM, they were overlaid with the culture medium for 12 days.

For culture in TGP, it was done as per our earlier studies. Briefly, a drop of the liquefied TGP which was reconstituted using the culture medium was placed in the centre of a 24-well tissue culture plate and was allowed to be solidified at 37°C for about 20–30 mins. Then an explant tissue was placed on the surface of the solid TGP and another layer of cold liquefied drop of the TGP- culture medium mixture was added to cover the gel with the tissue explant inside. Equal volume of the culture medium was added, and the tissue culture plates were placed at 37°C in CO2 incubator.

At the end of the 10th day, a portion of the cultures in TGP was harvested by placing the tissue culture plates for 1 h at 4°C–8°C to liquefy the TGP. Once the TGP is liquefied, the explant cultures were retrieved without the use of any enzyme.

3H thymidine incorporation assay

3H thymidine incorporation assay was used to evaluate the proliferative capacity of the cells. In this assay, the cells are incubated with 3H-thymidine for a few hours to overnight. Only proliferating cells can integrate the radioactive labelled thymidine into their nascent DNA and after the incubation period a scintillation counter is used to measure the uptake. In the current study, one μCi of 3H thymidine (BRIT, Mumbai, India) was incorporated into some of the wells of TGP and hAM cultured tissues. Fifty μL of medium from each well was sampled every day and relative consumption over ten days was evaluated. Beckmann LS 6500 scintillation system (Beckmann Instruments Inc, USA) was used for the 3H thymidine incorporation assay following the manufacturer’s protocol and following Sudha et al.

Harvesting cells from TGP for use in ex vivo attachment studies

A drop of cooled-liquefied fluorescent-conjugated TGP was placed in a new tissue culture plate and solidified at 37°C for about 20–30 min. The tissue explant with the cells
harvested for fluorescent-conjugated TGP studies was placed over the solidified TGP and another drop of liquified fluorescent-conjugated TGP was added to cover the gel with the explant and the cells over which cell culture medium was added and placed in at 37°C in 10% CO₂ incubator, when it became solidified to form a gel like scaffold for the tissues and cells to proliferate.

On the 12th day, the cells were harvested from all the TGP and hAM cultures. To obtain cells, the tissues harvested after liquifying TGP and by enzyme digestion from hAM. Then the cells were washed in cold PBS, pH 7.0, cyto-spinned (Cytospin 2, Shandon, UK) and placed on the microscopic slides for the histochemical staining studies.

A portion of the cells from TGP and hAM was used for transplantation onto cadaver cornea and another portion of the cultures was used for characterization studies. The fluorescent conjugated TGP cultures were washed with cold phosphate buffer saline (PBS) with florescent microscopy observation being performed before and after washing the TGP away.

**Histochemical staining**

Hematoxylin-eosin (HE) staining of the cultured explants in hAM and TGP were done according to standard protocols. Immunofluorescence staining and Immunoperoxidase staining for limbal stem cells’ marker expression was based on the work of Chen et al.28 and Sudha et al.21

For immunoperoxidase staining, after fixing with treated for 2 h at room temperature with primary antibody (p63, ABCG2, Connexin 43 and Integrin β), rinsed with Tris-HCl buffer, pH 8.0, incubation was done for 1 h with horse radish peroxidase conjugated anti-mouse secondary antibody at a dilution of 1:100. The substrate diamino benzidine (1:100) (DAKO cytation corp., Glostrup, Denmark) was purchased from DAKO Cytomation Corp., added, incubated for 5 min, rinsed, then counterstained for 30 s with Harris Haematoxylin.

For immunofluorescence staining, after washing with PBS-Tween 20, smears were treated with anti-mouse conjugated p63 (4A4 isofrom), ABCG2, Connexin 43, Integrin β with FITC (DAKO Cytomation Corp., Glostrup, Denmark) at 1:5 dilutions for one hour and counterstained with 0.5 percent Evans blue. The slides were glycerol-mounted and examined with a fluorescence microscope (Optiphot, Nikon, Japan).

Immunoperoxidase staining was performed on both cells from TGP and hAM cultures. Based on the findings, immunofluorescent staining was performed only on the cells from TGP.

**Ex vivo attachment of human corneal limbal epithelial cell to cadaveric cornea**

A donor cornea including the limbus was obtained from the eye bank and was placed in a sterile Petri dish. The limbal region was cut using sterile scissors and forceps to recapitulate limbal stem cell deficiency (LSCD). The corneal surface epithelium was removed by scraping, and then washed thrice with DMEM containing antibiotics (penicillin-streptomycin, amphotericin B, and gentamycin). A drop of liquefied TGP containing the LSC (pooled cells from different samples) cultivated in it was added to the corneoscleral rim, which was placed in a 24-well tissue culture plate. 500 µL of DMEM containing hAM’s F12 was added, and the tissue culture plate was incubated at 37°C in a 10% CO₂ incubator for 25 days with periodic observation.

**Results**

The donors’ ages ranged from 8–85 (mean = 59.25) years. The growth of LSC and corneal epithelial cells in TGP (Figure 1A, C) was microscopically observed as being better than the growth in hAM was (Figure 1B). Formation of a good monolayer was rapid from the cornea obtained from the 8-year-old eye donor compared to other donors. HE staining also showed better growth of cells in TGP compared to hAM (Figure 1D and E). The fluorescent-conjugated TGP study showed TGP was completely removed from the cells after washing (Figure 1F and G). Once the LSC–TGP mixture was added on to the cadaveric corneoscleral rim, the cells attached over it (Figure 2B). At 19 h post-transplant, the cells began proliferating from the centre of the denuded cornea (Figure 2C). Four days after the transplantation, cells proliferated at the limbal region with even better growth over the central cornea (Figure 2D). The cells continued to proliferate over the cornea in 25 days (Figure 2E). The incorporation of thymidine was rapid in the corneal tissues cultured within the TGP, on all the ten days of measurement. In the corneal tissue cultured in hAM, rapid incorporation of 3H Thymidine occurred only on the initial day (Figure 3). Immunoperoxidase staining showed positive for p-63 in both TGP and hAM (Figure 4A and E). Integrin β, ABCG2 and Connexin43 was positive in TGP (Figure 4B and C) but negative for cells cultured in hAM (Figure 4D-F). Immunofluorescence staining showed positive for limbal stem cell and corneal epithelial markers in the cells cultured from TGP (Figure 5).

**Discussion**

Autologous LSCT has been in practice for more than twenty years.29 A critical feature for success of the procedure depends on a proper three-dimensional environment to allow the cells to grow preserving their native phenotype and stem cell potency apart from using a good carrier material for transplantation.30,31 An ideal carrier material for LSCT should be malleable for easy handling during transplantation, transparent, biocompatible and facilitate corneal re-epithelialization and integration into the cornea.32 Several carrier materials including natural products such as hAM and synthetic scaffolds have been reported in earlier studies of LSCT.32,33 However natural scaffolds such as hAM have the disadvantages of biological risk of infection while the synthetic scaffolds have lesser biocompatibility than natural scaffolds.34,35 Acellular
matrices have also been reported as carrier materials, but they have disadvantage of lesser mechanical strength and malleability.35

The TGP used in the study has been earlier reported to provide a three-dimensional environment for the cells to grow along with preservation of the stemness of the cells cultured in it for a longer period.20 TGP is a transparent material,17,18 allowing visualization during in vitro culture and after the transplantation22 which has also helped in tracking of the cells by visual observation to document their

Figure 1. Proliferation of cells from corneal limbal explants in A. TGP; B: hAM, with better proliferation in TGP; C. Complete monolayer formation in TGP after 5 days; H and E staining of cells cultivated in D. TGP, E. hAM, F and G. Cell deposit from TGP observed under fluorescent microscope (50× magnification; blue filter = 450–490 nm) after four washings with cold saline. The fluorescein conjugate does not surround the cells, indicating the washings were complete (Scale bar = 100 μm).
expansion after ex-vivo transplantation, covering the ocular surface of a cadaver cornea. Cells after transplantation, once they get engrafted onto the surface of the epithelium, the TGP can easily be washed away by the tear fluid as proven in an earlier animal experiment which makes this a suitable material for efficient transplantation of cells onto a suitable surface of epithelium, as reported. These characteristics of TGP have potential applications in cell transplantations to epithelial surfaces as urethra, oesophagus etc. TGP’s biocompatibility has been earlier proven by in vitro and in vivo animal, human studies of a variety of cells including chondrocytes, bone marrow mononuclear cells, pluripotent stem cells and buccal mucosal epithelial cells. TGP allows for nutrition diffusion and is purely a synthetic scaffold, thus having an advantage over several other scaffolds.

The current study establishes the potential of TGP as a promising scaffold for corneal regeneration because it allows LSC cells to attach, proliferate, and form a confluent monolayer in 5 days and an epithelial sheet in 25 days. The growth in TGP was better compared to hAM in microscopic observation, 3H thymidine incorporation assay and histochemical staining (Figure 3–5). The attachment of the cells encapsulated into the TGP, on to the cadaveric epithelium and further migration, growth establishes TGP as an optimal scaffold for both in vitro culture and transplantation. The nutrition in this ex vivo study was from the DMEM containing Ham’s F12 medium, which was used to reconstitute the TGP and was overlaid during the evaluation of the corneal attachment on the denuded epithelial corneas of cadavers. This particularly establishes the nutrient diffusion capabilities of the TGP, which could be highly useful for tissue engineering and regeneration of avascular tissues such as the cornea and cartilage. In addition, fluorescence staining showed TGP could be easily washed away with the addition of PBS, which will be useful during in vivo transplantation (Figure 1E and F). In this case, the TGP will act only as a carrier material. The native corneal LSC phenotype was maintained during TGP culture with immunofluorescence staining and was positive for LSC markers. Immunoperoxidase staining showed positive for p-63 in both TGP and hAM Integrin β, ABCG2 and Connexin43 was positive in TGP (Figure 4) but negative for cells cultured in hAM. TGP scaffold has earlier proven to be a good cell-transplantation delivery material in both preclinical and human clinical studies. In in vitro studies, the amount of medium required for long-term culture needs further research. The present study also establishes the ex vivo

Figure 2. Phase contrast microscopic picture of (A) Corneal surface after removal of epithelial cells (black arrow indicates the cornea-scleral junction tissue which was cut all around to produce stem cell deficiency) (B) Cells over the corneal-scleral rim immediately after addition of the cell-TGP mixture onto the cadaver cornea (black arrow indicates growth of cells in the centre of the cornea) (C) 19 hrs after transplantation of the cell–TGP mixture (black arrow indicates the cornea-scleral junction) (D) 4 days after transplantation of the cell–TGP mixture; (E) 25 days after transplantation with corneal epithelium having proliferated over the surface of the cadaver cornea (Scale bar = 100 µm).

Figure 3. Results of 3H Thymidine incorporation assay of cells cultured in TGP and hAM, showing the rapid incorporation of thymidine in the corneal tissues cultured within the TGP, on all the ten days of measurement compared to those cultured in hAM.
culture platform of TGP encapsulated cells over a corneal tissue to serve as an *ex vivo* organ-culture model.

The major limitation of the study is that cells from explants grown only in TGP were transplanted over the corneoscleral rim while cells from explants in hAM have not been transplanted for side-to-side comparison. We are planning to study this in future studies. Another limitation being the evaluation of the number of cells transplanted versus that got engrafted, which will have to be studied under predetermined biological environments both *in vitro* and

Figure 4. Immunoperoxidase staining of cells cultured A-D: in TGP, positive for (A) p63, (B) Integrin β, (C) ABCG2 and (D) Connexin43; E-H in hAM, positive for (E) p63 but negative for (F) Integrin β, (G) ABCG2 and (H) Connexin43.
in vivo, because post-transplantation survival of cells depends on the nutritional availability of the in vivo environment.

As proven in this study, the capability of TGP further reinforces its utility as a multipurpose scaffold from basic in vitro cell culture as well as a carrier for encapsulation and transplant of cells to be delivered to the site where dysfunctional or damaged cells need to be replaced, followed by their engraftment. TGP is worth considering for similar applications that require cell transplant onto a surface of a tissue or onto the membranous surface of a lumen, or even within cavities of cell replacement within organs or for delivery of encapsulated cells of different germlines after necessary validation.

**Ethical approval**

The study was done in accordance with the declaration of Helsinki after Vision Research Foundation, Chennai, India’s institute ethics committee approval (IEC approval Code: 52 A-2004-P) for use of human ocular and amniotic tissues for cell culture and transplantation following national and international guidelines.

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**Disclosure statement**

1. Dr. Katoh is an employee of Edogawa Hospital, Japan and is an applicant/inventor to several patents on biomaterials and cell culture methodologies, some of them described in this manuscript.
2. Dr. Yoshioka is an employee of Mebiol Inc., and an applicant to several patents on TGP and its applications.
3. Dr. Abraham is a shareholder in GN Corporation Co. Ltd., Japan and is an applicant/inventor to several patents on biomaterials and cell culture methodologies, some of them described in this manuscript.
4. Other authors don’t have any conflict of interests to declare.

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