

Successful transportation of human corneal endothelial tissues without cool preservation in varying Indian tropical climatic conditions and *in vitro* cell expansion using a novel polymer

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Background: Though the transplantation of human corneal endothelial tissue (CET) separated from cadaver cornea is in practice, its transportation has not been reported. We report the successful transportation of CET in varying Indian climatic conditions without cool preservation and the *in vitro* expansion of Human Corneal Endothelial Precursor Cells (HCEPCs) using a novel Thermo-reversible gelation polymer (TGP). **Materials and Methods:** CET from cadaver corneas ($n = 67$), unsuitable for transplantation, were used. In phase I, CET was transported in Basal Culture Medium (Group I) and TGP (Group II) and in Phase II, in TGP cocktail alone, from three hospitals 250-2500 km away, to a central laboratory. The transportation time ranged from 6 h to 72 h and the outdoor temperature between 20°C and 41°C. On arrival, CET were processed, cells were expanded upto 30 days in basal culture medium (Group A) and TGP scaffold (Group B). Cell viability and morphology were documented and Reverse transcription polymerase chain reaction (RT-PCR) characterization undertaken. **Results:** In Phase I, TGP yielded more viable cells (0.11×10^6 cells) than Group I (0.04×10^6 cells). In Phase II, the average cell count was 5.44×10^4 cells. During expansion, viability of HCEPCs spheres in TGP was maintained for a longer duration. The cells from both the groups tested positive for B-3 tubulin and negative for cytokeratins K3 and K12, thereby proving them to be HCEPCs. **Conclusion:** TGP preserves the CET during transportation without cool preservation and supports *in vitro* expansion, with a higher yield of HCEPCs, similar to that reported in clinical studies.

Key words: Corneal endothelium, human corneal endothelial precursor cells, *in vitro* expansion, sphere forming assay, thermo-reversible gelation polymer, transportation

Cornea, the transparent outermost layer of the eye, is avascular, consisting of five layers the outer epithelium, Bowman's layer, corneal stroma, Descemet's membrane, and corneal endothelium. Corneal endothelium, the innermost layer, is a monolayer of mitochondria-rich cells derived from the neural crest;^[1,2] unlike the epithelium, it has limited regeneration capacity. The main function of the corneal endothelium is to control corneal hydration and nutrition^[3] and damage to the endothelium can affect corneal transparency, leading to corneal edema.^[4,5]

Currently, corneal transplantation is the treatment of choice, but there is a global shortage of donor corneas, which results in extended waiting times for corneal replacement surgery, especially in developing countries.^[6,7] In addition, in the present

situation, one donor cornea can be used to help only one patient recover vision.

In this scenario, the identification of precursor cells with regenerative capability and the methodology to expand those successfully *in vitro*^[8] will provide opportunities to develop potential solutions to corneal endothelial diseases.

Herein, we report our experience in transportation of human cadaver-derived corneal endothelial tissues (CETs) from different parts of India to a centralized facility, taking upto 72 h in varying climatic conditions using a novel thermo-reversible gelation polymer (TGP)-based transportation cocktail and the expansion of human corneal endothelial precursor cells (HCEPCs) in the TGP scaffold *in vitro*.

Materials and Methods

Thermo-reversible gelation polymer

TGP (Commercial name: Mebiol Gel) is a copolymer composed of the thermoreversible polymer (Poly (N-isopropylacrylamide-co-n-butylmethacrylate) (polyNIPAAm-Co-BMA)) and the hydrophilic polymer (poly ethylene glycol (PEG)).^[9] TGP remains in the fluid state at temperatures lower than the gel-sol transition temperature because of the water solubility of both TGP block and hydrophilic polymer block. It is transparent and the sol-gel transition temperature can be controlled by altering the

Access this article online

Website:

www.ijo.in

DOI:

10.4103/0301-4738.116457

Quick Response Code:



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Manuscript received: 30.01.12; Revision accepted: 15.10.12

chemical composition.^[10] TGP has been shown to support the growth of different cell types, including corneal limbal stem cells,^[11] Hepatocytes,^[12] Chondrocytes,^[13] continuous cell lines such as the Vero, HEp-2, HeLa, BHK-21, McCoy and CHO cell lines,^[14] embryonic stem cells, and induced pluripotent stem cells.^[15] Furthermore, TGP is a purely synthesized biocompatible polymer that has no biological contaminants. It has been found that TGP allows cells to grow in a three-dimensional form without differentiation for a relatively longer period of time compared with conventional scaffolds.^[13]

Preparation of transportation cocktail

The lyophilized TGP (1 gm) vial was obtained from Nichi-In Biosciences (P) Ltd, Chennai, India. The TGP was reconstituted with 10 ml of DMEM/F12 medium and incubated at 4°C overnight. Almost 200 µl of reconstituted TGP was added in a sterile vial and the basal culture medium containing 300 µl of Dulbecco's modified eagle medium: Nutrient mixture F-12 (DMEM/F12) (Gibco-BRL, Gaithersburg, Maryland (MD), USA) Epidermal growth factor EGF (20 ng/ml), Basic fibroblast growth factor bFGF (40 ng/ml) 0.3% Collagenase type-I (Gibco-BRL, Gaithersburg, Maryland USA) was overlaid on the TGP.

Phase I

Corneal endothelial tissues

All the procedures were in accordance with the declaration of Helsinki and approved by the institutional ethical committees of the hospitals involved in the study. Eight cadaver eyeballs were collected with proper informed consent from the guardians. The cadaver eyeballs were washed with iodine solution and then with sterile saline. The ages of the donors ranged from 13 years to 90 years. The corneal endothelial cell layer and Descemet's membrane were peeled away in a sheet from the periphery to the center of the inner surface of the cornea with fine forceps by experienced ophthalmologists as per an earlier described protocol.^[8]

Tissue transport

TGP was maintained below 4°C before the procedure, and four of eight CETs were immersed in the vials of the control medium containing 500 µl of basal culture medium, DMEM/F12 (Gibco BRL, Gaithersburg, MD, USA) culture medium containing

EGF (20 ng/ml), bFGF (40 ng/ml), and 0.3% Collagenase type-I (Gibco BRL, Gaithersburg, MD, USA) and this was considered as Group I [Fig. 1]. The remaining four samples were embedded in the cooled TGP vials and allowed to solidify at room temperature for five minutes. Around 300 µl of basal culture medium of composition described above was laid over the TGP. This was considered as Group II [Fig. 1]. Collagenase Type-I was added in the basal culture medium to facilitate the digestion process during the transport to reduce the time lapse between tissue harvesting and cell processing, as the CETs need to be subjected to overnight digestion before cell separation as described by Yokoo *et al.*^[8] The CETs were obtained from two hospitals (Hospital I and II described below) located at distances of approximately 250-300 km from the central processing laboratory, and the maximum time for transportation of Phase I samples was 12 h.

Phase II

In the Phase II study, the CETs were obtained from 59 cadaver eyeballs following the procedures described above. All the tissues were transported using only the TGP-based preservation cocktail described above.

The central laboratory was located in Chennai in the southern part of India, in the state of Tamil Nadu.

The locations of the hospitals from where the tissues were transported are as follows:

1. Hospital I: Approximately 250 km from the central laboratory
2. Hospital II: Approximately 300 km from the central laboratory
3. Hospital III: Approximately about 2500 km from the central laboratory.

The temperatures of the geographical areas between these locations are very varied, ranging from 20°C to 41°C depending on the season. The time of transportation to the central lab ranged between 12 h and 72 h.

CETs sample processing

Upon arrival in the laboratory, all the 67 specimens (Phase I (8 specimens) and Phase II (59 specimens)) were processed using the protocol described by Yokoo *et al.*^[8]

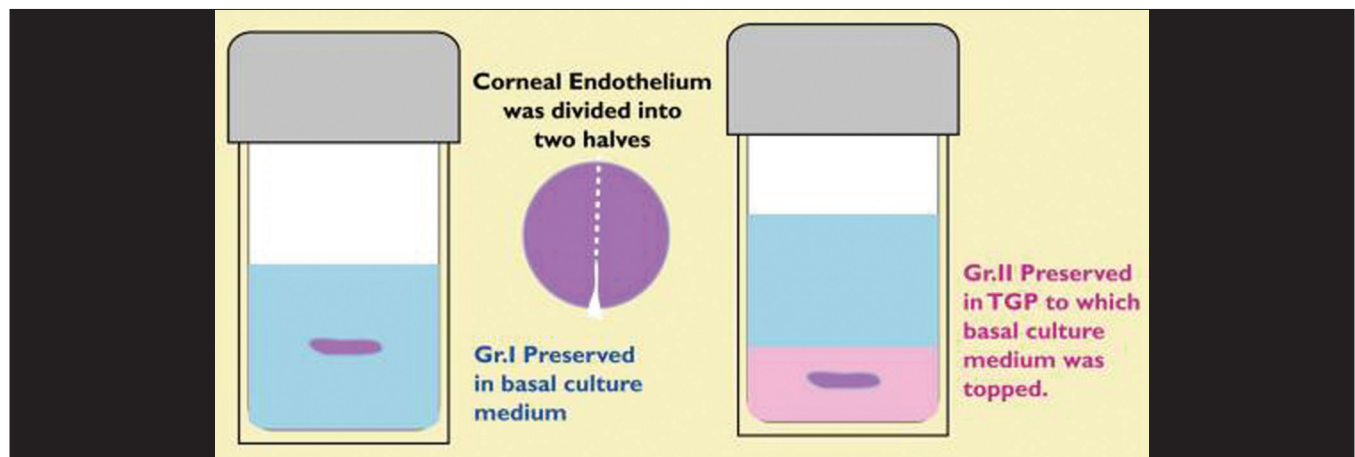


Figure 1: Schematic representation of the transportation methodology of the corneal endothelial tissues in two transportation cocktails as described in Phase I of the study

Briefly, the specimens were transferred into Poly HEME (Sigma, USA) coated 15 ml centrifugation tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and treated with 0.05% Trypsin EDTA (Gibco BRL, Gaithersburg, MD, USA) for 10 min at 37°C. After treatment with trypsin, 1 ml of basal culture medium containing DMEM/F12 (Gibco BRL, Gaithersburg, MD, USA) and 10% fetal bovine serum (FBS) was added for arresting the trypsin activity. The single-cell suspension was collected and filtered with a 70 µm sterile nylon mesh (BD, USA). Filtered single-cell suspensions were centrifuged at 2000 rpm for 10 min. After centrifugation, the pellet was collected and re-suspended in the basal culture medium. The cell viability was checked with Trypan blue exclusion method using Neubauer Chamber [Fig. 2]. The cells obtained from each sample were equally seeded into the following two groups.

Group A

A portion of the cells was seeded in 5 ml of the basal culture medium containing DMEM/F12 (Gibco BRL, Gaithersburg, MD, USA), EGF (20 ng/ml), bFGF (40 ng/ml), B-27 (Invitrogen-Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B in uncoated 12-well tissue culture (TC) plates (Corning Inc., Corning, NY) for floating culture and placed in a humidified incubator in an atmosphere of 5% CO₂ at 37°C.

Group B

The remaining portion of the cells was subjected to culture in a TGP-based culture protocol. Briefly, a drop of TGP (Mebiol Gel)-DMEM TC medium mixture was placed in the center of the 12-well TC plates (Corning Inc., Corning, NY) and solidified at 37°C. The cells suspended in the culture medium were placed over this solidified gel-TC mixture, following which a drop of the gel-TC medium mixture was again placed to cover

the cells. Thus, the cells were embedded within the TGP. The basal culture medium (DMEM/F12 (Gibco BRL, Gaithersburg, MD, USA) culture medium containing EGF (20 ng/ml), bFGF (40 ng/ml), B-27 (Invitrogen-Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B) was laid over the TGP. The culture plates were placed in a humidified incubator in an atmosphere of 5% CO₂ at 37°C.

The cells in both the groups were cultured for a maximum period of 30 days and observed for the formation of spheres or cell aggregations.

The spheres formed in Groups A and B were subjected to reverse transcription polymerase chain reaction PCR (RT-PCR) between the 14th and 30th day for the expression of the genes coding for neuron-specific marker β3-tubulin, and corneal epithelial stem cell markers CK3 and CK 12. The primer sequences used are presented in Table 1.

Results

In Phase I of the study, the cell counts obtained after transportation of eight samples in two groups of transportation cocktails are presented in Table 2. An average cell count of 0.04 × 10⁶ cells was obtained in Group I compared with 0.11 × 10⁶ cells in Group II. In Phase II of the study, 59 cadaver CETs were transported in the TGP-based transportation cocktail. The average count of cells after the sample transportation within 12 h was 5.24 × 10⁴ cells, whereas in the samples transported in 12-72 h, the cell count obtained was 5.64 × 10⁴ cells [Table 3]. There is no significant difference statistically between the 12 h (n = 21) transport group and the 12-72 h (n = 38) transport group.

During cell expansion, aggregation of cells as spheres was observed from the 12th day onward in Group A (Basal Culture medium without TGP) and from the 18th day onward in Group B (TGP-based culture). Group B exhibited a predominance of single cells forming spheres, whereas Group A exhibited aggregations of developing spheres. The spheres started degenerating/shrinking in size from day 24-28 in Group A, whereas their viability was well maintained in Group B even upto the 30th day. Sphere shapes were large but irregular in Group A [Fig. 3a], whereas spheres, though smaller in size, had a well-defined morphology in Group B [Fig. 3b]. Spheres from both the groups tested positive for the neuronal marker B-3 tubulin and negative for cytokeratins K3 and K12 [Fig. 4].

Discussion

Bullous keratopathy and Fuch's dystrophy are two common disorders of the corneal endothelium leading to loss of vision. Bullous keratopathy occurs due to abnormal corneal hydration and is characterized clinically by corneal opacification and the presence of epithelial vacuoles and bullae with swelling of the

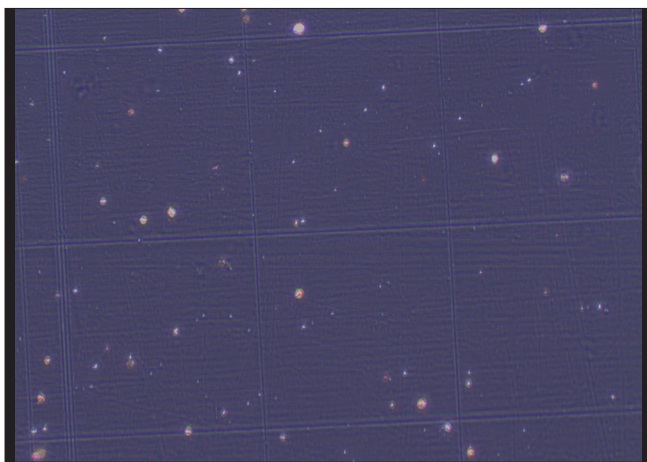


Figure 2: Corneal endothelial-derived cells immediately after transportation under an inverted phase-contrast microscope

Table 1: Primers used for polymerase chain reactions

Gene	Forward primer	Reverse primer
β3 Tubulin	5' TCTCACAAAGTACGTGCCTCGA3'	5' TGATGAGCAACGTGCCAT 3'
Cytokeratin K3	5' CATCATTGCTGAAGTTGGTGC 3'	5' TCTTGGAGCTTGGCATTGG 3'
Cytokeratin K12	5' TTGTGACAGACTCCAAATCA 3'	5' TACTCCAGTTGTCCAGAAGG 3'

corneal stroma.^[16] Fuch’s dystrophy is a slowly progressing degenerative disorder of the corneal endothelium with thickening of Descemet’s membrane, with similar corneal changes. Corneal transplantation is an effective approach to restore vision for conditions such as Bullous keratopathy or Fuch’s dystrophy.

Though the cornea is an immunoprivileged site conducive for transplantation of allograft corneas, earlier studies have reported that the success rate drops down to 74% at 5 years and 62% at 10 years. In high-risk cases with corneal neovascularization or ongoing ocular inflammation, the long-term 10 year survival rate is less than 35%.^[17]

Corneal endothelial cell-based approaches were considered to be difficult because the endothelial cells are prone to damage. Although it is not possible to expand these cells using standard culture techniques,^[8] the study by Yokoo *et al.* reported the isolation of corneal endothelial precursors by sphere-forming assay.^[8] A subsequent study on the injection of precursors derived from cultured human corneal endothelium into the anterior chamber in a rabbit model of corneal endothelial deficiency^[18] boosted hopes of an effective therapeutic cell-based strategy for corneal endothelial disorders. Cell-based therapies that employ the expansion of cells could offer potential solutions for the scarcity of human donor eyes because cells derived from one donor’s endothelial layer can be used multiple times, i.e., for possible use in more than one patient.

In this context, we wanted to study the feasibility of transportation of cadaver donor corneal endothelium from remote locations to a central processing facility where the cells could be expanded for use. The central processing laboratory is situated in the southern part of India and the three hospitals were located at a distance of 250-2500 km. Since a long duration of cold-chain preservation is not feasible in certain parts of India, the efficacy of a novel TGP-based transportation cocktail was assessed for transportation of CET specimens at varying outdoor temperatures ranging from 20°C to 41°C without cold-chain preservation. As an initial study, transportation in TGP-based cocktail was compared with transportation in a basal culture

medium. The results showed that transportation in TGP is equivalent to or better than basal culture medium in terms of cell viability (0.11×10^6 cells compared with 0.04×10^6 cells). Hence, a TGP-based medium was utilized for the transportation of 59 subsequent specimens. The viability of cells was maintained in the samples transported within 12 h as well as upto 72 h, with the average cell count being 5.44×10^4 cells.

Because CETs need overnight digestion, type I collagenase was added to the transportation cocktail itself to decrease the time lapse between transportation and processing, to preserve cell viability to the maximum extent possible.

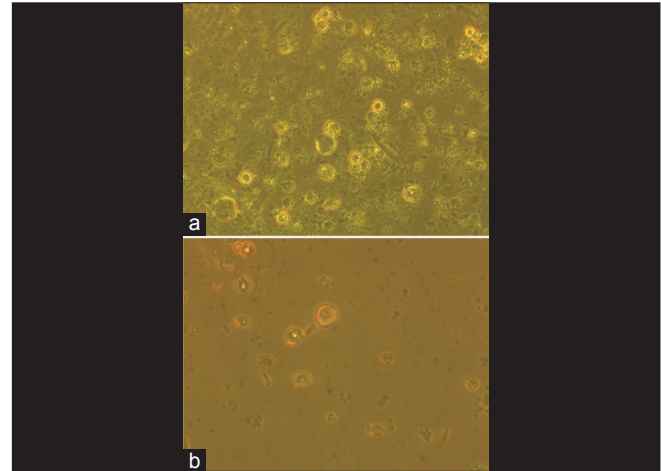


Figure 3: Corneal endothelial precursor cell spheres during *in vitro* culture: (a) In Group A (basal culture medium without Thermo-reversible gelation polymer (TGP)); (b) in Group B (cultured in TGP)

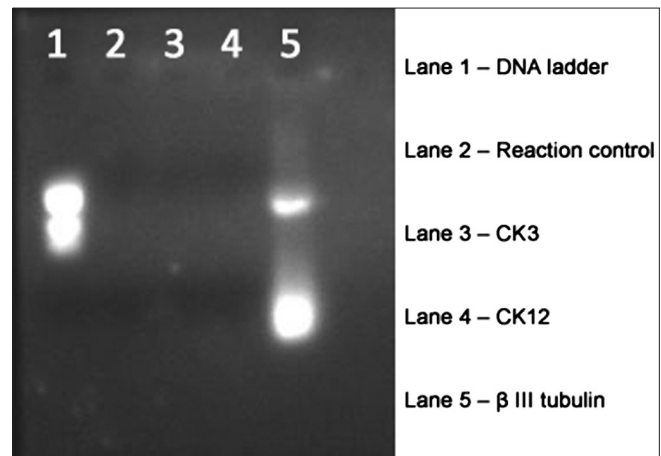


Figure 4: Reverse transcription polymerase chain reaction characterization of the *in vitro*-expanded human corneal endothelial precursor cells, showing positive markers for $\beta 3$ tubulin and negative for CK3 and CK12

Table 2: Post-transportation viability of corneal endothelial tissue-derived cells transported in two different transportation cocktails in phase I

Cell count of specimens transported in basal culture medium with TGP ($\times 10^6$)	Cell count of specimens transported in basal culture medium without TGP ($\times 10^6$)
0.14	0.006
0.12	0.01
0.12	0.1
0.06	0.075

TGP: Thermo-reversible gelation polymer

Table 3: Post-transportation corneal endothelial cell count of specimens transported in phase II study

Time duration of transportation	Number of specimens	Minimum cell count ($\times 10^4$)	Maximum cell count ($\times 10^4$)	Average cell count ($\times 10^4$)
≤ 12 h	21	1	22	5.24
12 h to 72 h	38	1	20	5.64

One of the major limitations of the study is that the baseline corneal endothelial cell counts could not be ascertained. The tissues were harvested from cadaver corneas, and during transportation, they were digested by incorporating collagenase in the transportation cocktail as described above. When the samples reached the central laboratory, they arrived as cells. Since the initial material of reference is a tissue and since each corneal tissue was divided equally into two portions, it is to be assumed that there were equal numbers of cells initially seeded in each of the two groups.

The expansion characteristics such as well-preserved sphere morphology with predominance of single cells forming spheres in a TGP-embedded culture (Group B) compared with suspension culture in basal medium (Group A), and the degeneration of spheres in Group A in contrast to the well-defined spheres in group B being maintained even upto 30 days without degeneration all demonstrate that TGP preserves cell morphology better than the conventional methodology used for processing and cell culture of HCEPCs. Furthermore, the RT-PCR method confirmed that the cells grown are indeed corneal endothelial precursors.

The scope of the present study was to assess the transportation viability of corneal endothelial cells and their successful post-transport *in vitro* expansion after a long duration of transportation in varying Indian climatic conditions. Therefore, we have studied the cells following exactly the same protocols as reported earlier after such transportation and subjected them to a limited number of vital and basic markers. In our future study using larger samples, we will study all the cell identification markers described in the article by Yokoo *et al.*^[8] Another limitation of the study is we did not incorporate 5-bromo-2'-deoxyuridine (BrdU) staining to distinguish spheres with viable proliferating cells from dying clusters of cells and this limitation will be overcome in a future study with a larger number of samples.

Earlier studies have reported that TGP is helpful in maintaining the three-dimensional culture pattern, which resulted in maintaining cell viability *in vitro* upto 90 days.^[13] In addition, the use of TGP as a scaffold for delivery of cells has shown better biocompatibility and engraftment without any toxicity.^[11] From our study, it is evident that the micro-architecture of TGP maximizes the corneal endothelial cell viability upto 72 h of transport without using any cool preservation methodologies. Moreover, TGP helps in maintaining the cell morphology and viability with the formation of healthy cell spheres even upto 30 days *in vitro*. This methodology will, thus, be useful for transporting cadaver CETs from any part of the world *via* airfreight with minimal cost and will also help in culturing the expansion of HCEPCs for therapeutic purposes.

Conclusion

In our study, a novel method for transportation of corneal endothelium in varying Indian climatic conditions without cool preservation has been accomplished in a reproducible manner, thereby avoiding the need for establishment of a current good manufacturing practice (cGMP) cell culture facility in each hospital. Instead, a central laboratory within a transportation time of 72 h can offer corneal endothelial cell processing and

expansion services to the eye hospitals situated in remote places too. The culture conditions for *in vitro* expansion of the HCEPCs without growth factors or feeder layers have been standardized, yielding cells that conform to the phenotype and genotype of those previously reported in clinical application. Therefore, these HCEPCs from one cadaver endothelial tissue could be used for cell therapeutics^[15] to treat many patient-eyes after *in vitro* expansion. These two accomplishments reported in the study could be of help in significantly reducing the backlog of patients with corneal endothelial diseases who await cadaver corneal endothelium, thereby minimizing the need for total corneal transplantation.

Acknowledgement

The authors thank M/S Hope Foundation (Trust), India for funding the study, M/S Chennai Cell Cluster for technical advice and Ms. Eiko Amemiya for her secretarial assistance.

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Cite this article as: Rao SK, Sudhakar J, Parikumar P, Natarajan S, Insaan A, Yoshioka H, *et al.* Successful transportation of human corneal endothelial tissues without cool preservation in varying Indian tropical climatic conditions and in vitro cell expansion using a novel polymer. *Indian J Ophthalmol* 2014;62:130-5.

Source of Support: The authors thank M/S Hope Foundation (Trust), India for funding the study, M/S Chennai Cell Cluster for technical advice and Ms. Eiko Amemiya for her secretarial assistance. **Conflict of Interest:** The authors Dr. Yuichi Mori and Dr. Hiroshi Yoshioka are share holders in the Mebiol Inc., Japan, manufacturers of one of the ingredients of the preservation cocktail.