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Citation	Current Trends in Biotechnology and Pharmacy, 2012, v. 6 n. 1, p. 44-54
Issued Date	2012
URL	http://hdl.handle.net/10722/191271
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Successful Transportation and *in vitro* Expansion of Human Retinal Pigment Epithelium and its Characterization; A step towards Cell-based Therapy for Age related Macular Degeneration

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Abstract

Age related Macular Degeneration (AMD) is a disease of the retina that leads to deterioration in vision and eventually permanent blindness. As yet there are no definitive ways of repairing the damage caused by AMD. Recently evidence is mounting that cell-based therapy using Retinal Pigment Epithelium (RPE) could be a feasible option for treating this disease. For example, autologous RPE transplantation has been successful at providing a functioning replacement for the diseased retina in animal models and humans. However, degeneration can re-occur requiring more RPE cells from the patient. Therefore, considering the option of one-time harvested RPE tissue from the periphery of the patient's eye, safe transportation between clinics/hospitals, efficient *in vitro* RPE expansion at the destination and long-term cryopreservation

for future applications, we have developed a biodegradable RPE carrying medium in 3D, made from a growth factor-free Thermo-reversible gelation polymer (TGP - Mebiol gel). RPE cell layers harvested from cadaver eyes were embedded in the TGP hydrogel and divided into three groups: Group 1, were processed immediately, Group 2 after 18-24 hours and Group 3 after 40-48 hrs of harvesting. Each group had one control sub-group grown in conventional media and one TGP sub-group grown embedded in TGP scaffold. No growth factors were used in the culture, when grown for three weeks. RPE cell counts were done at regular intervals during the expansion phase, and were then characterized by RT-PCR to confirm their RPE phenotype. The cells in all the three TGP preserved groups and the controls were equally viable after different periods of preservation, with a maximum

duration of 48 Hrs. In cultivation, TGP preserved RPE cells formed a monolayer with a typical honeycomb/cobblestone appearance characteristic of native RPE. The degree of pigmentation is increased in the TGP group compared to the control group indicating that the RPE possesses a native RPE phenotype. The proliferative capacity of RPE was also increased when embedded in TGP. Cells from both the groups expressed Cellular Retinaldehyde-Binding Protein (CRALBP) and RPE65, which are abundantly expressed in the RPE cells and Mueller cells of the retina. We have established a simple and efficient transportation module for RPE at varying climatic conditions without the need for cool preservation using a polymer hydrogel cocktail and a culture method without using any growth factors. These cells can be a potential source for transplantation in treating retinal disorders upon further confirmation of their functional characteristics.

Key words: Retinal pigment epithelium, macular degeneration, hydrogel scaffold.

Introduction

The Retinal Pigment Epithelium (RPE) is a neuroepithelium-derived, cellular monolayer that lies on Bruch's membrane between the photoreceptor outer-segments and the choriocapillaris. It serves to provide the transducing interface for visual perception (1) and hence is critical to providing vision (2). The RPE is also important for local homeostasis and maintenance of the extraphotoreceptor matrix.

Age related Macular Degeneration (AMD) is the third leading cause of worldwide blindness in the elderly that results in a loss of vision in the center of the visual field either due to atrophy of the retinal pigment epithelial layer, Dry Age related Macular Degeneration (Dry

AMD) or due to abnormal blood vessel growth, Wet Age related Macular Degeneration (Wet AMD) (3). The current treatments for wet AMD include: photodynamic therapy (PDT), laser therapy, anti-vascular endothelial growth factor (anti-VEGF) therapy etc., all of which have limited success and are non-permanent solutions. For dry AMD there are not many options available. The ideal treatment will be to replace the dysfunctional RPE cells by cell-based therapies which can repair the damaged retina and restore normal vision. RPE transplantation in humans was first performed in 1991 by Peyman *et al.* but with little success (4). Later allogenic fetal RPE cell transplantation was tried in which, immune rejection of the graft was a major issue. It was observed that rejection rates were lower in dry AMD than wet AMD (5). In many cases transplantation without Immunosuppression therapy led to leakage on fluorescein angiography and eventual fibrosis (6). All these lead to the era of autologous RPE transplantation which is conventionally done employing two techniques namely retinal pigment epithelial suspension and autologous full-thickness retinal pigment epithelial-choroid transplantation (7-11). Encouraging clinical outcomes has already been reported with the transplantation of the autologous RPE- Choroid from the periphery of the eye to a disease affected portion (10, 12, 13). Hurdles in autologous RPE transplantation include, the RPE cells being fragile when hit with surgical material, it is impossible to transplant a layer uniformly and in case of full-thickness transplantations including retinal pigment epithelial- Bruch's membrane- choroid transplantation, formation of multilayered folds and contraction of the graft pose as major hurdles (14). Also the option of autologous graft is constrained by the size of the full thickness RPE graft that can be taken from the periphery of the same eye to patch the defect

and this is usually insufficient. As a result, an alternative source of cells to replace the diseased RPE is needed (15). For this, we believe that a safe, reproducible and efficient *in vitro* expansion of the human RPE could be developed for the clinic. Hence, there arises the need for a scaffold material that will support RPE cells while culture similar to a Bruch's membrane, helping in fluid transport with porosity and helps in transplantation of RPE in the sub-retinal space and is bio degradable. Recent approaches employ the use of substrates like amniotic membrane (16 - 18). Singhal *et al.*, has grown RPE cells obtained from human cadaver eyes on human Amniotic Membrane (*hAM*) as culture substrate and reported that the Primary adult human RPE cell cultures retain epithelial morphology *in vitro* when cultured on human amniotic membranes. However after the expansion, separation of the RPE cell from amniotic membrane and transferring of the expanded RPE cells without contamination of amniotic membrane is difficult. The use of feeder layers and growth factors are another disadvantage, as a source of contamination and immunoreactivity in the host.

Herein, we report the transportation of RPE tissue layers in a novel polymer based transportation cocktail from hospitals to a central processing facility without cool preservation under varying climatic conditions and subsequent isolation, expansion and characterization of the RPE cells in a synthetic biodegradable polymer scaffold without use of growth factors.

Materials and Methods

Isolation of Retinal Pigment Epithelium:

Twelve RPE samples obtained from eyes of human cadavers after proper informed consent from the guardians were used in the study. These RPE samples were obtained from three different hospitals located at varying distances of 300 to

3000 kms from the central processing facility taking a maximum time of 48 hrs. 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.

The RPE tissues were dissected and embedded in Thermo Reversible Polymer based cocktail by an experienced ophthalmologist.

Preparation of Transportation cocktail: The transportation cocktail consisted of TGP (Commercial name: Mebiol Gel) which is a copolymer composed of thermo-responsive polymer blocks [poly(N-isopropylacrylamide-co-n-butyl methacrylate) poly(NIPAAm-co-BMA)] and hydrophilic polymer blocks [polyethylene glycol (PEG)]. The TGP is in liquid state at lower than the sol-gel transition temperature but turns to gel immediately upon heating and returns to a liquid state again when cooled. This sol-gel transition temperature can be altered (19). The lyophilized TGP (1gm) vial was provided for this study by Nichi-In Biosciences (P) Ltd, Chennai, India. The TGP was reconstituted with 10 ml of DMEM/F12 (Gibco BRL, Gaithersburg, MD, USA) and incubated at 4°C overnight. 200 µl of reconstituted TGP was added in each sterile vial and the basal culture medium which need to be overlaid contained 300µl of DMEM/F12 culture medium without growth factors.

Transportation of RPE in TGP based cocktail :

The TGP vial was taken from the refrigerator and the RPE tissue was embedded in the TGP in the vial over which additional TGP kept under cold condition was added and left undisturbed for five minutes for the cold TGP to solidify. After solidification of TGP, basal culture medium described above was overlaid and the tissue

containing vials were transported to the central processing facility without cool preservation. One RPE tissue was transported in one TGP vial. Thus the RPE tissues embedded in TGP vials were divided into three groups, Group 1, Group 2 and Group 3. RPE tissues in Group 1 were those tissues which were processed within 18 hrs of transport to the central processing facility, Group 2 contained RPE tissues which were processed after 18-24 hours and Group 3 contained those which were processed after 40 – 48 hours.

Tissue Processing: For tissue processing, the RPE tissue transported in TGP vials were kept in cold condition for the TGP to liquefy. All the RPE tissues were subjected to washing by adding cold Calcium Magnesium free CM-HBSS with antibiotics (penicillin 50 U/ml, Streptomycin 0.1 mg/ml and Amphotericin B 0.0025 mg/ml) and centrifugation at 1200 rpm for 10 minutes at 4 p C using an ultra low temperature centrifuge. After centrifugation, the supernatant was discarded and the pellet was washed twice. The washed RPE tissue pieces were subjected to enzymatic digestion with 0.25% of Trypsin and 0.02% of EDTA for 10 minutes. After 10 minutes of incubation, equivalent amount of Dulbecco's Modified Eagle's medium (DMEM)/Ham F12 medium with 10% FBS was added to neutralize the Trypsin activity. The cell suspension was collected and centrifuged at 1200 rpm for 5 minutes. Cell count was done using Trypan Blue Dye exclusion method. The cells obtained were seeded equally in two groups for *in vitro* expansion.

Control Group: The cell pellet was mixed in Dulbecco's Modified Eagle's medium/Ham F12 medium supplemented with 10% Fetal Bovine Serum and antibiotics (penicillin 50 U/ml, Streptomycin 0.1 mg/ml and Amphotericin B 0.0025 mg/ml) in 24 well culture plates.

TGP Group: The cell pellet was plated in 24 well

culture plate containing TGP. Additional TGP kept under cold conditions was added on top of the cells and they were left undisturbed for 5 minutes for the cold TGP to solidify. DMEM/ Ham F12 medium supplemented with 10% Fetal Bovine Serum and antibiotics (penicillin 50 U/ml, Streptomycin 0.1 mg/ml and Amphotericin B 0.0025 mg/ml) was overlaid on TGP after solidification.

Both the groups were cultured for 21 Days in 5% CO₂ at 37°C and documented. Periodical adding of culture medium and observation were done in the same manner for both groups of specimens.

Cell count and characterization of cultured Retinal Pigment Epithelium: On the 21st day the cells were harvested and subjected to cell counting using trypan blue exclusion method and documented. The cultured RPE cells from treated and un-treated groups (Control Group and TGP Group) were subjected to H&E staining and RT-PCR analysis. The total RNA content was isolated from cultured Retinal Pigment Epithelial Cells. Gene expression for major proteins specific to the native RPE was confirmed using RT-PCR specific primers such as RPE 65 which is a RPE specific 65 kDa protein abundantly expressed in the RPE cells and Mueller cells of the retina (20) and Cellular Retinaldehyde-Binding Protein (CRALBP) which is a 36-kD water soluble protein and carries 11 cis-retinaldehyde or 11 cis-retinal as physiological ligands (21). GAPDH was used as control.

Results

The viability of the RPE tissues were well maintained after transportation in the TGP based preservation cocktail, and we could obtain an average of 0.91 x10⁶ cells after preservation for a duration of 12- 48 hrs and the duration of preservation did not have any statistically

significant effect on the viability of the cells. The Cells counts obtained after processing and culture for 21 days are depicted in Table-1. The RPE cell proliferation was higher in the TGP group in majority (83.3%) of the specimens (Graph 1). The TGP group had a 2.38 fold increase in cells on an average whereas the control group had a 2.17 fold increase (Graph 2). RPE Cells in TGP Group formed a monolayer, showing a typical honeycomb appearance (Fig.1) whereas in the Control Group these characteristics were not observed. The cells in the Control Group exhibited Glial cell like morphology (Fig. 2), whereas in TGP Group the cells resembled neuronal like spheres (Fig. 3). The expression of pigments was better in TGP Group compared to Control Group (Figs. 4, 5).

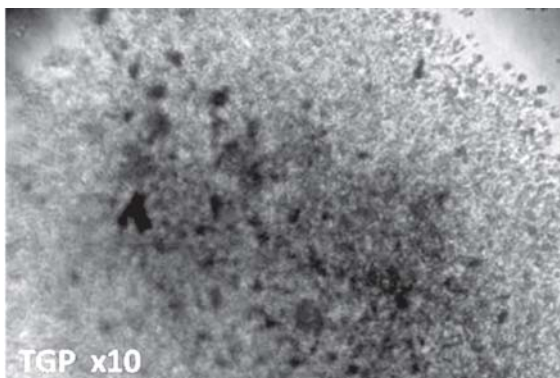


Fig. 1. *In vitro* expanded RPE cells in TGP group showing honey comb morphology

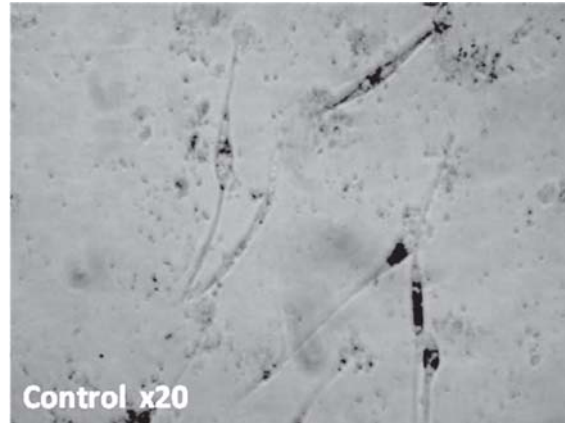


Fig. 2. RPE cells in control group shows Glial Like cells *in vitro*

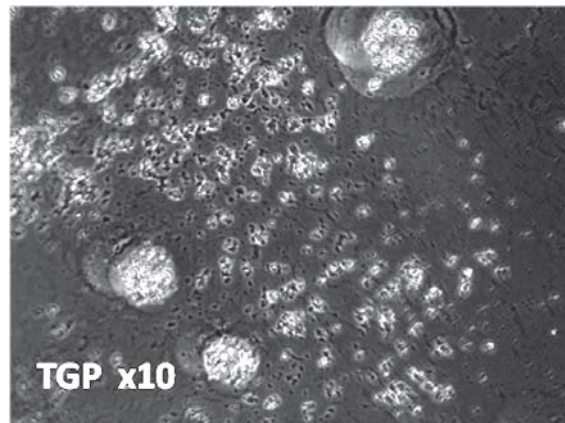


Fig.3. *In vitro* expanded RPE cells in TGP group exhibiting neuronal like spheres

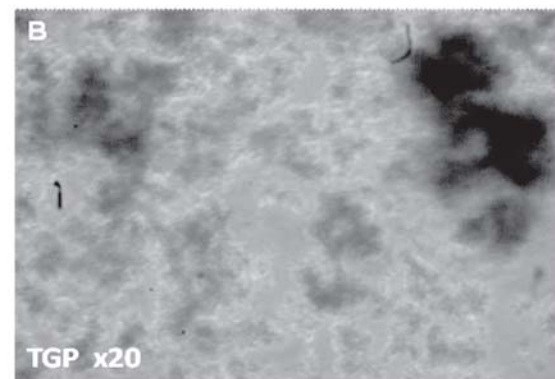
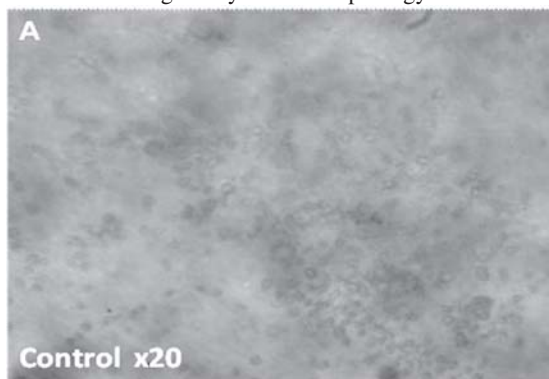


Fig. 4. RPE cells showing relatively more pigmentation in TGP group (4B) when compared with control group (4A) after 21 Days

Histologically (H & E staining), dark pigmented epithelial cells were observed in both the groups, confirming the *in vitro* expanded cells to be RPE cells. Large cells with large nuclei suggestive of immature neuronal cells were observed in the TGP group (Fig. 6). RT-PCR showed the expression of the retinal pigment epithelial (RPE) cells specific markers, Cellular Retinaldehyde-Binding Protein (CRALBP) and retinal pigment epithelial 65 (RPE65) in both the groups (Fig.7).

Discussion

The World Health organization (WHO) states AMD to be the third leading cause of visual impairment with nearly 9% of the world population affected by this disorder (22) and the prevalence is expected to double by 2020 (23).

Current therapeutic approaches to AMD include thermal laser photocoagulation, surgical approaches like excision or displacement, photodynamic therapy and more recently anti-vascular endothelial growth factor (VEGF) therapies. However all these therapies aim to minimize visual loss and optimize vision-related quality of life (14). The search of an ideal therapeutic approach that would help in regaining complete vision, still continues in which, the approach of RPE transplantation holds great promise. Autologous RPE transplantation faces hurdles such as (i) insufficient quantity of cells in the graft and (ii) risk of biological contamination with use of feeder layers, animal derived growth factors for cell expansion and biological scaffolds like amniotic membrane which have

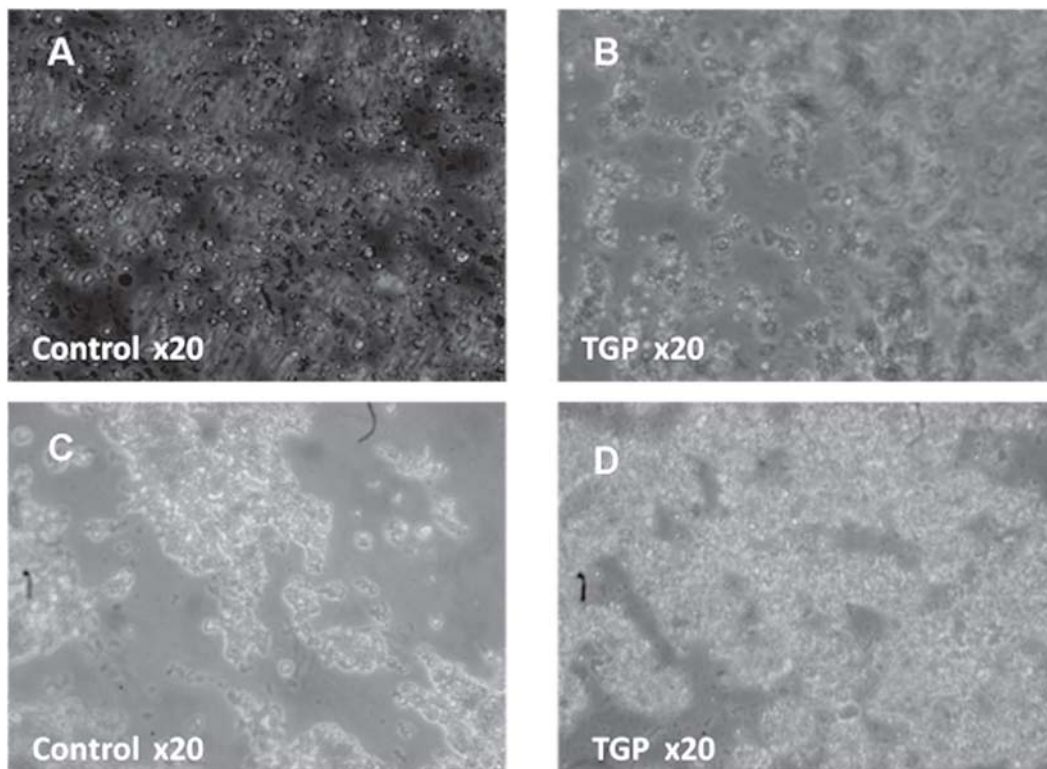


Fig. 5. Inverted microscope images of the RPE cells during *in vitro* expansion: A, B (Day 7) and C, D (Day 14)

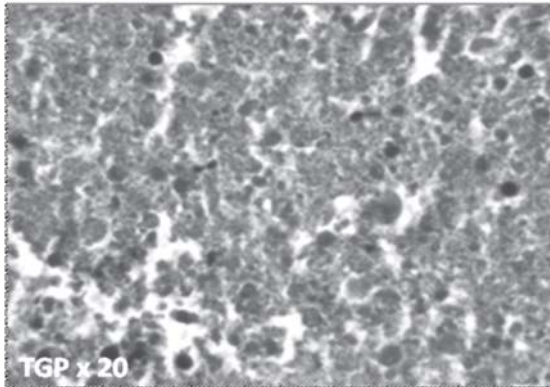


Fig. 6. H&E staining of the *in vitro* expanded cells show scattered dark pigmented epithelial cells. Large cells with large nuclei suggestive of immature neuronal cells are also seen.



Fig. 7: RT-PCR characteristics of the *in vitro* expanded RPE Cells. Lane 1: 100 bp Ladder; Lane 2(control) : GAPDH and CRALBP ; Lane 3 (TGP) : GAPDH and CRALBP; Lane 4(control) : RPE65; Lane 5(TGP): RPE65

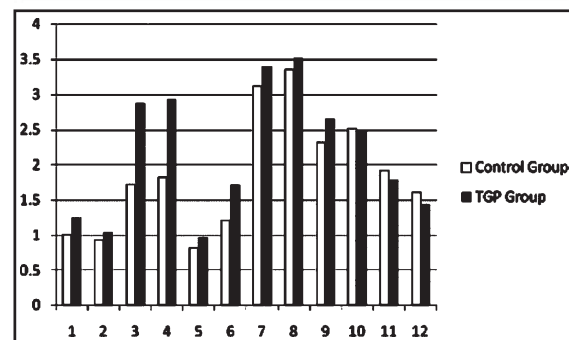
an inherent threat of contamination after transplantation. To our knowledge, an ideal scaffold to support RPE cells for *in vitro* expansion and to act as a carrier for transplantation has not yet been reported. We

have accomplished the successful transportation of RPE tissues in a novel Thermo-reversible Gelation polymer (TGP) based transportation cocktail from remote hospitals to a central processing facility without cool preservation under varying climatic conditions and using the same polymer as a scaffold, subsequent isolation, expansion and characterization of the human retinal pigment epithelial cells without use of growth factors.

We performed this study using TGP as a 3D scaffold for transportation and cell expansion *in vitro* because TGP has shown to support the culture of several cell types including corneal

Table 1. Cell count after *in vitro* expansion for 21 Days

S. No	Initial cell count	Cell Count after <i>in vitro</i> expansion for 21 Days	
		Control Group	TGP Group
1	0.71	0.99	1.25
2	0.85	0.93	1.04
3	0.96	1.71	2.88
4	1.05	1.82	2.93
5	0.61	0.81	0.97
6	0.86	1.2	1.72
7	1.15	3.12	3.4
8	1.09	3.35	3.52
9	1.44	2.32	2.65
10	1.06	2.51	2.48
11	0.64	1.92	1.78
12	0.57	1.6	1.44

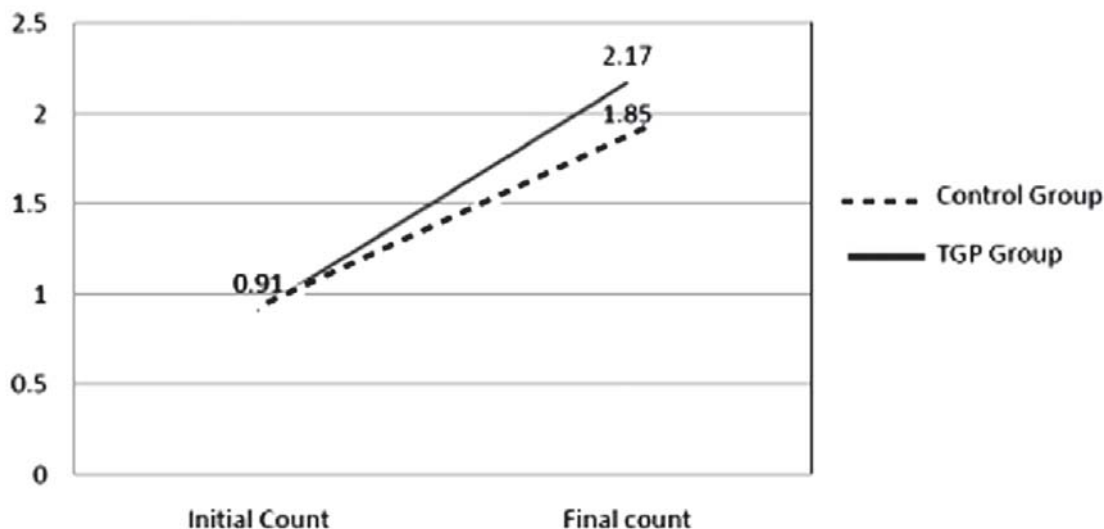


Graph 1. Comparison of RPE cell count after *in vitro* expansion in Control Group vs TGP Group after 21 days

limbal stem cells (24), Hepatocytes (25), Continuous Cell lines (26), chondrocytes (27), neural cells (28) etc. TGP has already been proven to preserve the cell viability of corneal endothelial cells after transportation in varying climatic conditions without cool preservation (29). In this study, this result has been replicated for RPE transportation. In addition cell viability of the RPE tissues was maintained after transportation in TGP and these cells could be expanded substantially *in vitro* which is evident by the higher fold increase of cells in the TGP group compared to control group (Graph 2). The transportation without cool preservation would prove valuable in developing nations like India where cold chain preservation in remote areas is difficult. Another major obstacle faced in the culture of RPE is the loss of phenotype or de-differentiation of the pigmented cells resulting in loss of pigmentation. This loss of pigmentation has been observed after sub-retinal transplantation of the cultured RPE too (30). In the present study, the ability of TGP to maintain the phenotype of cultured RPE was confirmed

by the presence of neuronal spheres in TGP Group which were not observed in Control Group. Also the presence of intracellular pigments was greater in TGP Group cultures compared to Control Group. TGP provides a three-dimensional tissue culture environment that helps the cells to grow for longer period of time without losing their viability (31). In many studies on RPE culture, 3T3 feeder layers are employed. Johnen *et al.* reported that when RPE was cultured on 3T3 fibroblasts, RPE cells exhibited stable expression of pigment epithelial genes, but expression of mouse collagen type 1 was also observed (18) which is a disadvantage for clinical translation.

The methodology employed in the present study eliminates the need for growth factors or feeder layers. When TGP is used as a substrate for RPE cell culture and transplantation into the sub retinal space, it could be advantageous because the separation of cells from the substrate is easy. There is no need for enzymatic digestion to separate the cells due to the unique sol-gel



Graph 2. Comparison of Percentage of increase in RPE cell count between Control Group and TGP Group after 21 Days

transition property of the TGP, which reverts into liquid state when it is cooled. TGP has been previously used by Sitalakshmi *et al.* in the transplantation of corneal limbal stem cells into the eyes of rabbits (24). TGP is biologically inert (32) and biodegradable (33). Thus, TGP satisfies most of the properties that is required for successful RPE transplantation. This study has established the utility of TGP for transport of RPE tissues in a viable manner and its efficacy to support RPE tissues in culture. Further studies are needed to compare the functional characteristics and post-transplantation efficacy of allogenic RPE Vs autologous RPE transplantation cultured in TGP scaffolds in animal models before a clinical transplantation. Sub-retinal space being an immunoprivileged site, the allogenic RPE transplantation if proven to be successful without any adverse reactions would provide a simple and attractive new solution. Thereby, the RPE tissues, which are discarded once the corneal button is harvested from cadaver eye, could be an indispensable source of RPE cells for treating the AMD of many patients.

Conclusion

We have established a simple and efficient transportation modality of human cadaver donor derived RPE tissues at varying climatic conditions without cool preservation embedded in a hydrogel cocktail and a methodology for *in vitro* expansion of the RPE cells without using any growth factors. RPE cells showed better growth characteristics in TGP even after transportation at varying climatic conditions for 48 hours suggesting that TGP can be used as a suitable substrate for both transportation and *in vitro* expansion. The *in vitro* expanded RPE cells using TGP could be a potential source of RPE transplantation procedures for treating Age related macular degeneration after confirmation of their functional efficacy in animal models.

Acknowledgement

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

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