Original Article

A proper protocol for isolation of retinal pigment epithelium from rabbit eyes

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Abstract

Background: Retinal pigment epithelium (RPE) is a hexagonal monolayer of pigmented cells located between the neural retina and the choroid with an essential role for visual function. So, isolation, propagation and maintenance of their functional integrity of RPE are crucial for research *in vitro* which next used for cell transplantation. The evaluation of features of RPE cells as a sheet after 14 days has not been reported yet. This study aimed to examine and compare three protocols for RPE isolation from rabbit eyes and obtain a proper protocol, which illustrated isolated RPE cells as a sheet cause to preserve their characterize even after 2 weeks.

Materials and Methods: RPE cells were prepared from eyes of 24 rabbit eyes. After enucleating of eyes, anterior segment discarded and posterior segment cut to small pieces. Two of these procedures are based on the enzymatic digestion, but third protocol based on mechanical dissection. The culture cells harvested and morphological feature of cells assessed by phase-contrast microscope and then analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunocytochemistry.

Results: Evaluation of morphological feature showed that isolation of RPE cells as a sheet lead to preserve their hexagonal morphology. Immunocytochemistry and RT-PCR assessment demonstrated RPE cell cultured in sheet maintained their phenotypic feature, tight junction and the distribution of actin and cytokeratin filament. Comparison of three protocols showed that dissociation of RPE cells as a sheet was superior in the preserve of RPE characteristic.

Conclusions: Isolation of RPE cells as a sheet maintains the integrity of these cells, this procedure promising a therapeutic approach, which is important for some retinal diseases.

Key Words: Cytokeratin 18, enzymatic digestion, retinal pigment epithelium, sheet, zonula occludens-1 protein

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INTROUDUCION

The retina is the innermost of the three concentric layers of the eye. It consists of two basic layers: Neural retina and retinal pigment epithelium (RPE).

The RPE is a monolayer of cuboidal cells that rest on Bruch's membrane. RPE cells are connected by a

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junctional complex consisting of gap junction, elaborate zonulae occludentes and adherents. This junctional complex is the site of the "blood-retina barrier."[1] The RPE justifies many tasks that are crucial for visual function[2] including: (A) Transports of nutrients such as glucose or vitamin A, from the blood to the photoreceptors,[3] (B) removal of accumulated water from the subretinal space due to metabolic activity,[4] (C) maintenance the immune privilege of the eye,[5] (D) facilitates visual process by re-isomerization of all-trans retinal into 11-cis retinal[6] and maintenance of outer segment by phagocytiosize of shed outer segment of photoreceptors.[7] In addition, RPE cells release several neurotrophic growth factors for maintenance of the functional integrity of the photoreceptors like pigment epithelium derived factor,[8] which protects retinal neuron against hydrogen peroxide-induced cell death,[9] basic fibroblast growth factor that effects survival and morphological differentiation of photoreceptor cells.[10] Therefore, the RPE cells are essential for the survival and function of photoreceptors and dysfunction of one of these activities can lead to degeneration of the retina, impairment of visual function and blindness.

Degeneration of the neural retina results in a number of retinal disease including age relative macular degeneration that originate from RPE degeneration[11] or retinitis pigmentosa, a group of inherited disorders that associated with progressive loss of photoreceptors or retinal pigment epithelium (RPE).[12] These retinal defects can be correctable by several therapeutic approaches, among which, transplantation has been proposed as the most promising treatment. On the other hand, there is no successful treatment for the retinal disorders caused by RPE degeneration. RPE cells are the main source for cell replacement. In addition, these cells can be used for *in vitro* evaluation of new therapies. Hence, suitable procedures are necessary to replace disorder RPE with healthy RPE. The quality of the RPE cells, which preserve their characters are most important criteria for transplantation. Contamination of RPE with choroid cells, loss of properties and reduction in their function are the limitations of these cells. In order to overcome these limitations, we should use an effective method to isolated RPE cells. So, to achieve this aim, the technique of RPE cell preparation is a determining factor.[13-16] There are several methods for isolation of RPE cells from choroid and neural retina.[17-20] Therefore, isolation, propagation and maintenance of their functional integrity of RPE are crucial for their transplantation. However, limited studies were carried out on the isolation of RPE cells as a sheet, which in these studies, RPE sheet triturated to single cells, but we maintained RPE cells as a sheet for 2 weeks. To our knowledge, examination of properties of RPE cells as a sheet after 14 days and comparison with these two methods has not been reported. In the current study, we compare three protocols for RPE isolation. Two of these procedures are based on enzymatic digestion while the third protocol is based on mechanical.

MATERIALS AND METHODS

Animals

Eyes were obtained from 24 pigmented rabbits that weighed between 1.5 and 2.0 kg. All animal care, surgical processes were undertaken in strict accordance with the approval of the Ethical Committee of Royan Institute. Pigmented rabbits were sacrificed by an overdose of ketamine and xylazine. Then, the globes were enucleated and washed in Ca^{2+} and Mg^{2+} -free phosphate buffered saline supplemented with penicillin/streptomycin (GIBCO, 15140-0122).

Isolation and culture of rabbit RPE

First protocol

The anterior segment, lens and vitreous were discarded and posterior part incubated overnight in RPE medium. The RPE and choroid were incubated for 45 min at 37°C in a collagenase IV 1 mg/ml (Invitrogen,17104-019), then incubated in trypsin (GIBCO, 25300-056) 0.1% for 35 min. After naturalized the enzyme reaction, cells harvested with RPE culture medium.

Second protocol

In accordance with protocol of Engelmann and Valtink[13] using minor modifications, after enucleating of eyes, circumferentially, the incision of the globe was made 3 mm posterior of the limbus. The anterior segment was discarded, lens and vitreous removed. Then, posterior segment cut to small pieces and these parts incubated 4 h in a mixture of collagenase I and IV (0.5 mg/ml). After stopping of enzyme reaction by RPE culture medium (DMEM/F12 [GIBCO, 31331-028], 10% FBS [GIBCO, 10270-106], 0.1 mμBME [SIGMA, M7522], 0.1% NEAA [GIBCO, 11140-035], 0.1% LGLU [GIBCO, 25030-024], 0.1% amphotericin B [SIGMA, A2942]) we used pure sperm gradient to separate RPE cells easily. Then, triturate pellet cells incubated in medium F99 (Medium 199 [SIGMA/M2154] nutrient mixture Ham's F12 supplemented by 1% fetal calf serum [FCS]), for 4 days at 37°C under 5% CO2 atmosphere. After 4 days, cells harvested and the supernatant is stored at –20°C. In this experiment, the following growth medium based on choroid-conditioned medium: 10% (conditioned for 4 days in F99 + 1% FCS), FCS: 10%, insulin: 1 mg/ml (bovine), antibiotics at recommended concentration.

Third protocol

By gaining advantage from Chang *et al.*,[15] with some reformation, eyes were enucleated from anesthetized rabbits and cleaned of extraocular tissue. The intact globe washed in Ca^{2+} and Mg^{2+} free phosphate buffered saline supplemented with penicillin/streptomycin. Then, the globes were incubated in 2% dispase (Gibco, 17105-041) for 20 min. An incision was made in the globe just 3 mm posterior of the limbus and extended circumferentially. After remove of anterior segment and making four radial incisions in the posterior segment, these part incubated in DMEM/F12 supplemented with 10% FBS for 2 h. Finally, the RPE cells were separated from the neural retina and choroid as a sheet with micropipettes and under a stereo microscope (Olympus, SZX16).

Immunocytochemistry staining

immunofluorescence staining was performed through fixation of RPE cells with 0.4% paraformaldehyde in PBS and permeabilized with 0.4% Triton X-100. For blocking of non-specific sites, we used 10 mg/ml bovine serum albumin. Then cells were incubated overnight with primary antibody (Zo-1 Invitrogen, 330100 and Cytokeratin 18 Chemicon, MAB3234). Labeled cells were detected with either FITC- or TRITC-conjugated secondary antibodies for 1 h, between all incubations, cells were washed with PBS 3-5 times, 5 min each wash. Nuclei were stained with DAPI (Sigma, D8417) and observed under a fluorescent microscope (Olympus BX51, Japan) equipped with an Olympus DP70 camera.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

For assessment of natriuretic peptid receptor-A (NPR-A) (F: AGAGGGAGAACCTGACCAACCG R: ACGATTCTGGAATTCCTGATACTCG) expression as rabbit RPE marker in three protocol, total ribonucleic acid was extracted and digested with, random hexamer primers and RevertAid™ first strand complementary deoxyribonucleic acid (Fermentas, K1622). PCR reaction was performed using SmarTaq polymerase and NPR-A primer. Amplification conditions were as follows: Initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, extension for 45 s at 72°C and a final polymerization at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel to prove that the suitable expected sized product was produced.

MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay

In order to determine the rate of cell viability in isolated RPE cells as a sheet, we use MTS assay. Briefly 2×10^5 RPE cells were cultured in 96-well tissue culture plates under RPE medium. Two weeks later, cell viability assay was performed by MTS/PMS (Promega, G5421) according to the manufacturer's protocol. Absorbance was measured at 492 nm by the enzyme-linked immunosorbent assay reader.

Statistical analysis

Data were analyzed by one-way ANOVA followed by least significant difference *post hoc* with a significance threshold of $P < 0.05$.

RESULTS

Maintenance of hexagonal morphology

Comparison of phase-contrast microscopic appearance of the three protocols showed that mechanical isolation and culture of RPE cells as a sheet maintains the hexagonal morphology of these cells [Figure 1]. On the other hand, these microscopic images suggest that after 14 days in this model, the morphology of RPE cells are better preserved than the enzymatic method.

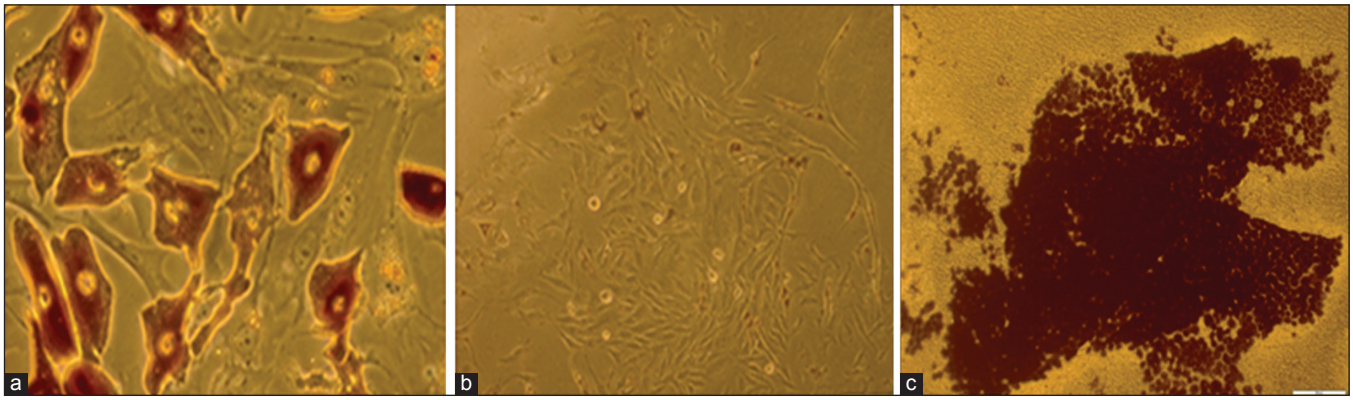

Figure 1: Phase contrast photomicrographs of retinal pigment epithelium-isolated cells showing cellular morphology according to enzymatic methods 1 (a), 2 (b), and the mechanical method (c). Scale bar: 20, 50, 100 μm, respectively

Expression of cytokeratin 18 and ZO-1

In the cultured isolated RPE, the immunocytochemistry images showed that cytokeratin 18, an intermediate protein was expressed in the RPE sheets; in contrast this protein was not expressed in two other protocols [Figure 2]. In addition, staining with anti-ZO-1 antibody, tight junction protein, we demonstrated that tight junctions remain intact in sheet cultures of RPE cells [Figure 3].

RT-PCR analysis

Expression of NPR-A, rabbit RPE marker was confirmed by RT-PCR in cultured of RPE sheet [Figure 4], this marker was absent in the enzymatic protocols. This expression was maintained over 14 days of culture [Figure 5].

MTS result

In addition, MTS assay demonstrated that these cells as sheet maintain their metabolic activity even after 14 days of culture. The mean absorbance for MTS assay on day 0 was 1.25 ± 0.33 and on day 14 was 1.31 ± 0.25 [Figure 6] and their difference was not significant at $P < 0.05$.

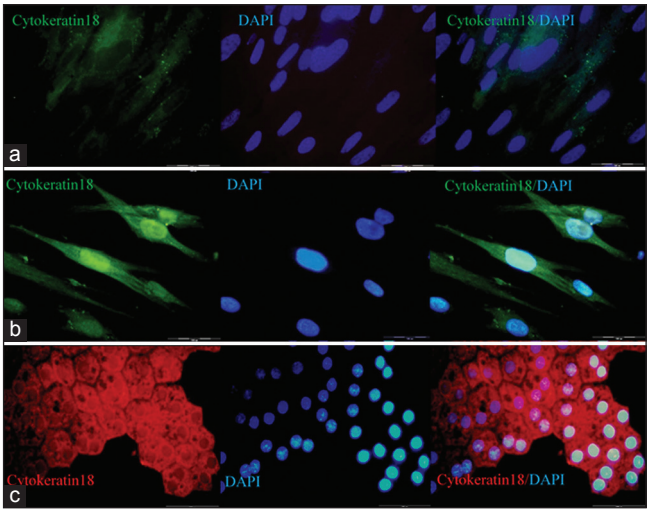

Figure 2: Immunocytochemistry staining of retinal pigment epithelium cells demonstrates expression of cytokeratin 18 in the mechanical protocol (c), which was not expressed in the enzymatic protocols (a and b)

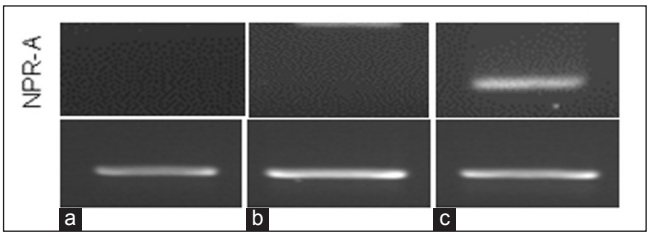

Figure 4: Reverse transcriptase-polymerase chain reaction analysis showing the expression of rabbit retinal pigment epithelium marker NPR-A in the mechanical protocol (c)

DISCUSSION

The first efforts to culture of RPE cells were described about three decades ago.[13] Consequently, different techniques to isolate RPE cells have been described[21-23] including enzymatic and mechanical isolation of RPE cells. Motohiro Kamci *et al.*[14] obtained and cultured retinal pigment epithelial cells as a sheet with proteinase K and evaluated wound healing as a RPE cell function. Likewise, Lidan Cong *et al.*[24], also used dispase to obtain RPE cells as a sheet, but triturated into single cells and transplanted to subretinal space of rabbit eyes. However, in our study, the sheet was kept 2 weeks in medium culture and it was observed that the cells conserved their properties. In the enzymatic method collagenase is used to separate RPE cells from basement membrane. Engelmann and Valtink isolated the RPE/choroid complex with incubated tissue in a mixture of collagenases IA and IV,[13] here in this study we modify this protocol and use pure sperm gradient to remove choroid layer. Most studies investigated the effects of RPE-choroid layers in contact with neurosensory retina.[25,26] We previously revealed the

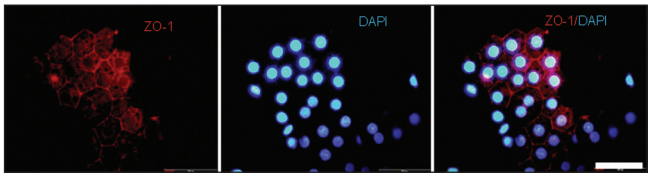

Figure 3: Retinal pigment epithelium isolated by the mechanical protocol illustrates expression of tight junction protein ZO-1

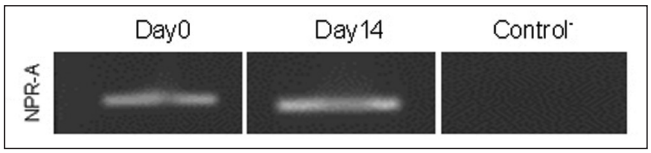

Figure 5: Reverse transcriptase-polymerase chain reaction analysis indicatingthe expression of the specific protein, natriuretic peptid receptor-A on days 0 and 14 in the mechanical protocol

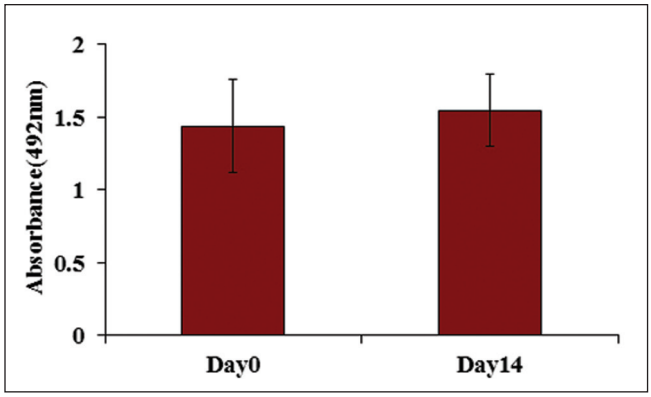

Figure 6: Comparison of the mean absorbance of retinal pigment epithelium in the third protocol on days 0 and 14 after culture ($P \leq 0.05$)

differentiated effects of pure RPE cells as a sheet in the co-culture system with retinal progenitor cells.[27]

In this report, we examined and compare three techniques to isolation of RPE cells. In each one, the most effective process is to discard the anterior segment, vitreous and retina and separate RPE/choroid complex. In the initial step or the protocol one, the separated RPE/choroid were treated with collagenase type IV and then singled by trypsin. Our result demonstrated that RPE isolated in this procedure did not maintain their morphological appearance and did not express specific protein like cytokeratin 18 or the NPR-A. This observation may partly relate to contamination of RPE cells with choroid cells. In the second protocol, which was base according to Engelmann and Valtink in addition to enzymatic digestion by collagenase I and IV, the cell suspension were layered over pure sperm gradient to remove cell contaminates. In addition, the separated cells after 4 day of culture were further cultured in condition medium obtained from choroid cells. Similar to the first procedure, despite cell proliferation within the culture medium, the cells did not maintain their integrity and did not express cytokeratin 18 and NPR-A. Therefore, in the third protocol, the RPE were mechanically separated, as sheet from choroid layer following treatment eye globe with dispase and were culture for 14 days. The results revealed that the sheet maintain their cellular integrity and maintain their expression of cytokeratin 18 and NPR-A. They also maintain their metabolic activity shown by MTS assay. MTS assay also revealed that these cells when in the form of sheet do not proliferate. Another superiority of the latter method is that the cells maintain their pigmentation and hexagonal morphology and express ZO-1. Maintenance of cell pigmentation has not been reported for the first two procedures. The only short come of this procedure, is that the cells do not proliferate in this method and therefore, the method might not be suitable for cell transplantation, however is appropriate for research where function RPE layer is required for co-culture with other cells, such as neural precursor cells. One aim of this method is evaluation the quality of RPE cultures prior to transplantation by cognition of specific markers.

In conclusion, the results of this study reveal that when RPE are separated as a sheet, they main their integrity and retain their RPE characteristic, which is important for promoting research *in vitro*.

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