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An improved method for the isolation and culture of retinal pigment epithelial cells from adult rats

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Glossary and Abbreviations

AMD/ ARMD: Age- related macular degeneration: degeneration of the macula, which is the part of the retina, at an advanced age.

BRB: Blood- retinal- barrier: part of the blood - ocular barrier that consists of retinal cells that are joined tightly to limit certain substances the movement between the retinal capillaries and the retinal tissue.

Bruch's membrane: is the border between the retina and the choroid. It is formed by the basal membranes of the RPE and the vessels of the choriocapillaris and contains several layers of collagen and elastic fibres.

Choriocapillaris: highly fenestrated blood vessels at the apical border of the choroid.

Confluence: 100% confluency means the surface of the culture disc is completely covered by the cells.

DMEM: Dulbecco's modified Eagles‘medium. Cell culture medium that can use to maintain cells in tissue culture.

DMEM-medium: Culture medium with DMEM, 10% FCS and 1% Pen Strep.

EPON: A type of epoxy resin used in sample preparation for electron microscopy**.**

FBS/FCS: Fetal bovine serum/ fetal calf serum. Most widely used growth supplement for cell culture media because of its high content of embryonic growth factors.

Fibroblasts: Type of cell that is responsible for the synthesis of the extracellular matrix and collagen. These cells are the most common cells of the connective tissue in animals.

FITC: Derivative of fluorescein used for fluorescence labeling of biochemical substrates.

Lipofuscin: Age pigment, which accumulates in lysosomes of postmitotic cells with highly oxidised undegradable material.

Long evans rat: Pigmented rat (Long Evans, Charles River, Sulzfeld, Germany).

Melanosomes: Cells that are responsible for synthesis, storage and transport of melanin. They are synthesized in the skin melanocytes, choroidal melanocytes and retinal pigment epithelial cells.

Microvilli: Microscopic, finger-shaped evaginations that cause an increased surface area and are involved in functions like absorption, secretion or cellular adhesion.

Neubauer chamber: A hemocytometer used to count cells.

OsO_4: Osmium tetroxide, Osmium(VIII) oxide.

Ora serrata: A round and serrated borderline between the retina and the ciliary body.

Pen Strep: Penicillin Streptomycin. Antibiotics, used to prevent bacterial contamination of cell culture due to their effective combined action against gram-positive and gram-negative bacteria.

Phagocytosis: Ingestion of extracellular material by a cell; here: ingestion of retinal photoreceptor outer segments by the RPE; the endocytic vesicle is called Phagosome.

PBS: Phosphate buffered saline. (+: with calcium and magnesia, -: without calcium and magnesia)

Photoreceptors: Light - sensitive cells (rods and cones) that are found in the retina of the eye.

ROS: Retinal photoreceptor outer segments: they form the apical parts of rods and cones, which receive light from the lens and convert it into electric signals. In rods, the visual pigment is called rhodopsin. It can be used for the immuno-localisation of ROS after phagocytosis by RPE cells.

RPE: Retinal pigment epithelium: epithelial monolayer of neural tube origin; together with the endothelial cells of the choriocapillaris it builds the blood-retinal barrier between choroid and retina.

RPM: Revolutions per minute. Measure of the frequency of rotation.

Tight junction: Intercellular junction between epithelial cells that separates proteins localized in the apical and basal part of RPE cells.

Triton X-100: Non-ionic surfactant, which dissolves membrane proteins out of their original conformation. Here: to permeabilize the cells before immunostaining.

Trypan Blue: A stain that is actively extruded from viable cells, but which readily enters and stains dead cells.

Trypsin: Enzymatic reagent that is used to release adherent cells from tissue culture plates for passaging.

Wistar rat: Albino rats (Wistar, Charles River, Sulzfeld, Germany).

ZO-1: Zonula Occludens-1, Tight junction protein.

Die Ergebnisse dieser Arbeit sind bereits publiziert und zum Teil wörtlich übernommen. Zur Kenntlichmachung der übernommenen Passagen wurden die Textabschnitte in Kursivschrift und mit der Quellenangabe [3] markiert.

I. Introduction

1.1 Structure of the RPE

The retinal pigment epithelium (RPE) is a highly polarized, pigmented single layer of hexagonal cells located between the choriocapillaris and light- sensitive outer segments of the photoreceptors, in the background of the eye. The RPE is part of the retina and of neuroectodermal origin. It plays a central role in the maintenance of visual function by close interaction with the outer segments of the photoreceptors [1].

The RPE cells have a honeycomb appearance, which is described as the most solid shape of cells in nature. The largest coverage of area without cell overlap or empty area is allowed by the hexagonal structure of the cells [2].

A RPE cell consists of an apical portion adjacent to the photoreceptors with microvilli and a basal portion on Bruch`s membrane [3]; [4]. This cell- building with basal infoldings and apical microvilli serves as enlargement of the surface. Tight junctions together with the blood vessels of the choriocapillaris build the blood-retinal barrier, which prevents the entrance of toxic molecules and plasma components into the retina and controls the crossing of fluids and solutes across the blood-retinal barrier. This sealing function plays an essential role in the integrity of the retina [1].

The histology of the RPE is shown in figure 1.

This structure maximizes cell- cell communication, which plays an important role regarding the numerous functions of the RPE, which are redescribed in 1.2.

With normal aging, the morphology of RPE cells underlies several changes, whereas little is known about these changes. The understanding of the normal aging process of RPE is subject of experimental investigations and will better enable us to understand differences in age related retinal pathology [5].

The RPE cells are cubical, highly polarized and densely packed with pigment granules. Within the first two postnatal weeks a significant proportion of the cells become binucleated in vivo and in vitro [6].

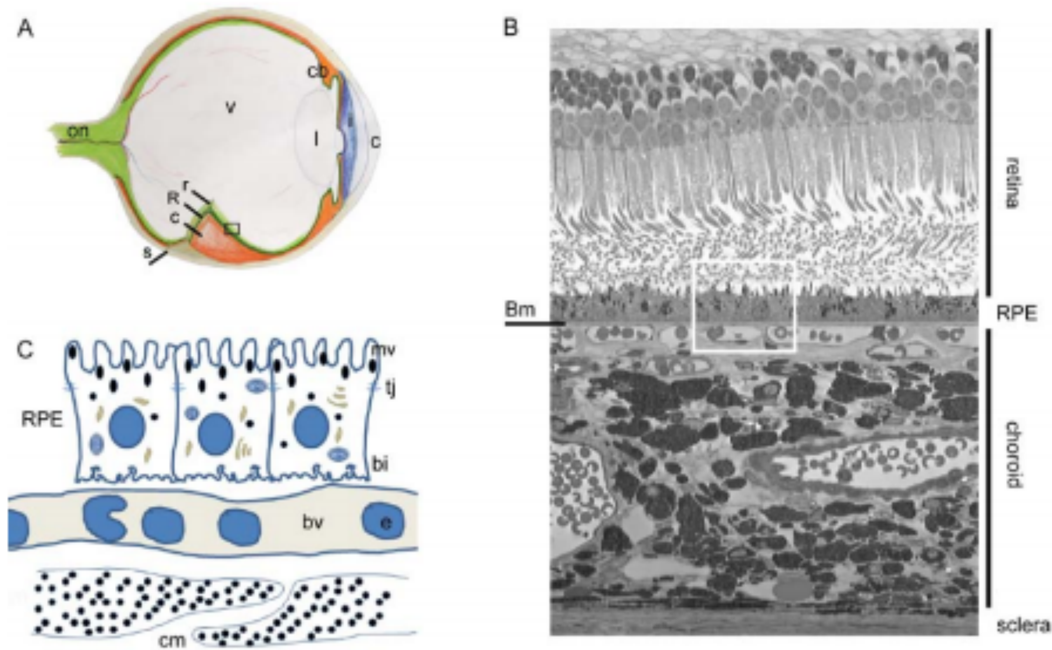


Figure 1

Histology of the retinal pigment epithelium (RPE) A) A scheme of the vertebrate eye shows the RPE (R) lying between the retina (r) and the choroid (c); sclera (s), c cornea, cb ciliary body, i iris, l lens, on optic nerve, v vitreous body; the highlighted square is magnified in B.

B) Cross section of a monkey eye: the apical part of the RPE is closely related to the photoreceptors of the retina. Bm Bruch's membrane;

C) Schematic diagram of the RPE, as highlighted in B: basal infoldings (bi) and apical microvilli (mv) enlarge the surface; tight junctions (tj) of the RPE together with the blood vessels (bv) of the choriocapillaris build the blood-retinal barrier; choroidal melanocytes (cm) are densely packed with round melanosomes (black spots), while RPE melanosomes are more oval shaped, e erythrocyte; [7].

1.2 Functions of the RPE

The RPE cells accomplish several functions essential for vision in the healthy retina.

1. First the RPE is an essential part of the blood-retinal barrier and mediates the bidirectional transport between the neural retina and the blood vessels in the choroid [1]. So the RPE guarantees the balanced nutritional supply of the retina by letting nutrients and oxygen through. Thus, it transports ions, water, and

metabolic end products from the subretinal space to the blood [1]. *This function is carried out by the formation of tight junctions between the RPE cells, preventing free diffusion of large molecules from the choriocapillaris to the neural retina [8]; [9]; [10]. The close contact to the neural retina and underlying highly vascularized choroid makes the RPE a critical point of application for different drugs. Systemically administered drugs may evoke toxicological complications by breaking through the blood-retinal barrier and having an effect on the retinal pigment epithelium [8]; [11]; [12]; [13].*

2. *One of the most important functions of the RPE is the phagocytosis and degradation of retinal photoreceptor outer segment discs (ROS), which are shed during the light perception process [14]; [15]; [16]. At the early stage of 12 - 15 postnatal days, the RPE cells already take up the phagocytosis process* [5]; [17].
3. Another function of the RPE is related to their melanin content [18]. The melanosomes of the RPE absorb scattered light, which would otherwise disturb visual acuity [19] and, melanosomes protect against oxidative stress in the background of the eye [20].
4. Another pigment, lipofuscin, gradually accumulates in the cells of the retinal pigment epithelium [21]. It contains non - degradable remnants of phagocytosed photoreceptor outer segments [22]; [23].
5. In addition, the RPE is able to secrete sundry growth factors, important for the maintenance of structural integrity of photoreceptors and choriocapillaris endothelium. This secretory activity, also plays an important role for the immune response of the eye [1].

1.3 Diseases of the RPE

If any of these functions are disrupted, it can lead to loss of visual function, degeneration of the retina and ultimately to blindness. Diseases, for example such as age- related macular degeneration (AMD) can cause a dysfunction or death of the RPE cells, but also drugs can damage these cells. Other examples of diseases that affect the posterior eye segment are diabetic retinopathy, glaucoma, retinal venous and arterial occlusive diseases, inherited retinal

degenerations and posterior uveitis. The most common of these diseases is AMD.

1.3.1 Age- related macular degeneration (AMD)

AMD is a malfunction of the eyes macula, a small area in the retina which contains the highest density of photoreceptors and represents the light-sensitive tissue lining the back of the eye. As part of the body`s natural aging process, many older people develop macular degeneration.
Symptoms can be such as blurriness, dark areas or distortion in your central vision, and perhaps permanent loss of your central vision. When macular degeneration does lead to loss of vision, it usually begins in just one eye, though it may affect the other eye later. There are different kinds of macular problems, but the most common is age-related macular degeneration. There are two types of AMD: the dry, or atrophic macular degeneration and the wet macular degeneration, whereas the dry form is more widespread. [24]
RPE cell transplantation currently plays a major role in eye research, especially as a treatment for AMD.

1.4 Aim of this study

To analyse the influence of drugs on the RPE makes it necessary to cultivate RPE cells in vitro.
RPE cells have been successfully isolated from human [25]; [26]; [27], bovine [28]; [29]; [30], and frog eyes [31], but in the rat and mouse this is either limited to neonatal animals [32]; [33] or when from adult mice, then they are not appropriate for long-term cultures [34]; [35]. The attachment of rat RPE to Bruch's membrane is very strong and makes it difficult to brush off the RPE without damaging the cells. In addition, the strong interdigitation of RPE apical microvilli with the ROS makes the separation of these two structures problematic in adult rodents. By using young animals (less than 15 days old), when the ROS are not fully developed, RPE cells can be isolated without tearing away their apical microvilli. Another critical point known from the culture of human RPE cells is that

once cultured, the adult RPE cells typically lose their hexagonal morphology along with other key functions over time [36]. Cultured RPE cells change to fusiform morphology and lose their ability to phagocytose ROS. As RPE cells divide, their pigment becomes diffuse and is often released from cultured cells over time. Associated with the assumption of the fusiform morphology is the loss of adherent junction-related proteins, known to be essential for an actin filament-based epithelial phenotype [36]. Thus, it is essential to maintain in vivo morphology over long-term culture.

Since no satisfactory procedure exists to isolate RPE from adult rats, the goal of this study was to find out a reproducible and quantifiable method for the isolation and culture of RPE cells from adult rats. To this aim, three methods of culture were performed and compared in terms of yield, cell survival, and morphological characteristics. Moreover, potential differences between RPE cells originating from pigmented versus albino rats were investigated. [3]

In addition, the purpose was to determine the effect of a test substance in terms of cell viability, phagocytic activity, the effect of repeated ROS feeding-hypertrophy, cell polarity and protein synthesis. The purpose behind these experiments was to test if method 3 is workable for drug testing. The test substance itself was not the focus of this work.

We also followed up to the difficulty, to overcome the retinal adhesion of RPE microvilli to rod outer segments.

II. Materials and Methods

Three methods are described, whereas each method was developed from the same initial preparation process until the eyecups were separated from the neural retina.
The main difference was that the RPE cells in method 1 remained on Bruch`s membrane whereas in methods 2 and 3 they settled on the plastic bottom of the culture dish. [3]

2.1 Animals

Pigmented rats (Long Evans, Charles River, Sulzfeld, Germany) and albino rats (Wistar, Charles River, Sulzfeld, Germany) were used for these experiments. They were maintained on a 12L: 12D circle in accordance with the ARVO Statement for the Use of Animals in Research.
A total of 180 pigmented rats and 340 albino rats at the age of 6-14 weeks were used. Rats were anesthetized with a ketamine- xylazine cocktail (100 mg/kg ketamine (WDT, Garbsen, Germany), 5 mg/kg xylazine (WDT)) and killed by decapitation using a guillotine. [3]

2.2 Enucleation

Eyes were enucleated under sterile conditions and after removing the residual connective tissue, they were directly incubated for 1 minute in 70% ethanol in order to eliminate fibroblasts on the outer surface of the eye. Then the eyes were rinsed twice with PBS (-) and incubated in PBS (-) overnight at 4°C to reduce the adhesion between the neural retina and the pigment epithelium. The eyes were again immersed for 1 minute in 70% Ethanol. All subsequent dissections were carried out under sterile conditions, by using a dissecting microscope, under a light source, with the eyes submerged in DMEM. The fluid environment is important for the tissues, because they should not be allowed to desiccate. The dissection of albino tissues is performed on a dark mat to optimize the colour contrast [3]; [31].

2.3 Separation of the retina from RPE

Up to the point of a slight modification, the micro dissection was carried out according to Edwards [32]. The eyes were opened by incision just posterior to the ora serrata by using a sharp copped scalpel and the anterior part was dissected by a circumferential section. The cornea, lens and vitreous were discarded by forceps. To overcome the damage to the RPE by removal of the retina, four different approaches were compared of removing the retina without harming the RPE cells:

1. *Direct mechanical removal after enucleation, without any further treatment*
2. *Soaking the eyes overnight in Ca free PBS.*
3. *Soaking the eyes overnight in DMEM at 4°.*
4. *Pre - treatment with Trypsin in different concentrations for varying times.*
5. *Pre - treatment with enzyme solutions of hyaluronidase and collagenase directly as described by Wang et al. [34]*

The most effective method was the third approach. The retina was carefully removed after cutting of the optic nerve with a small punch. The detached retinas were immediately fixed for electron microscopy, in order to check if the RPE was damaged or had adhered to the retina during the dissection. For the subsequent procedure, there are three different methods of culture of the retinal pigment epithelium. Figure 2 a and b show the eyecups from pigmented and albino rats submerged in DMEM before further preparation [3].

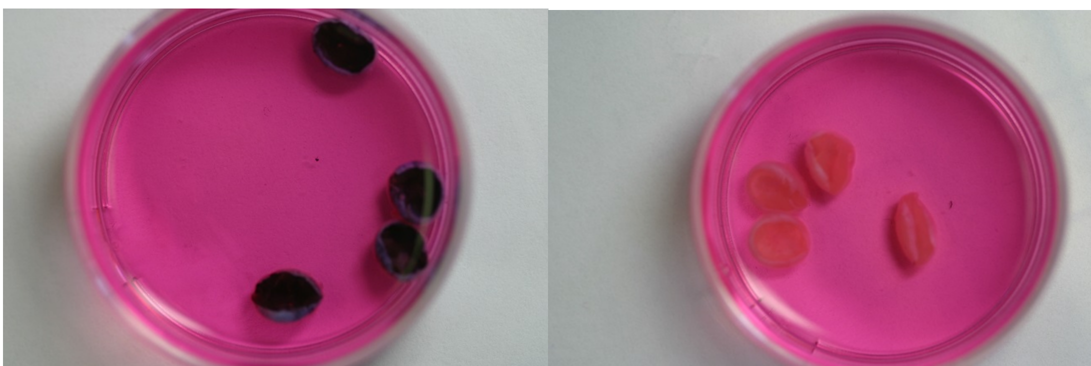


Figure 2 a **Figure 2 b**

2.4 Methods for RPE isolation and culture

2.4.1 Method 1: Growing directly on flat mounts

Three radial incisions were made into with a curved scalpel so that the eyecup was sliced into six cloverleaf-shaped components consisting of sclera, choroid and RPE. These flat mounts were submerged in 0, 5 ml DMEM medium with 10 % FCS in 24-well plates and stored at 37°C and 5% CO_2 in the incubator. The medium was used for all 3 methods and replaced completely three times a week. The RPE cells were inspected at different points of time in culture (immediately after isolation (day 1) and after 2 and 4 weeks) under a fluorescence microscope. For this purpose, the pieces of tissue were covered on a slide with fluorescent mounting medium (Dako, Glostrup, Denmark) and carefully embedded with a cover slip. Figure 3 shows six flat mounts from a pigmented rat after the preparation process [3].

Figure 3

2.4.2 Method 2: Enzymatic method

Four eye cups were placed in a small petri dish and 0,125% Trypsin solution (Böhringer, Mannheim) was added into the cups. The petri dishes were put into the CO_2-regulated incubator for 10 minutes at 37°C. Then by pipetting some jet streams of enzyme solution onto the surface of the retinal pigment epithelium the RPE cells were separated from Bruch`s membrane. The enzyme reaction of trypsin was stopped after 10 minutes by adding DMEM containing 10% FCS (fetal calf serum) and the cell suspension was centrifuged 5 minutes at 1500 RPM. The supernatant was discarded and the cells were resuspended in DMEM under sterile conditions. Aliquots of the cell suspension were incubated with 0.5 % trypan blue solution in PBS and counted in a Neubauer chamber. The cell

suspension was then plated into multi-dish 96-well plates (Nunc, Roskilde, Denmark) using a cell quantity of 15000-20000 cells/well. Cells were also plated in 8 well glass chamber slides (Lab-Tek, Nunc, Rochester USA) at the same density to compare the results. Cells were incubated in a CO_2 incubator in a humidified 95% air / 5% CO_2 atmosphere and they were observed every second day by phase-contrast microscopy. The culture medium was changed completely three times a week, for a period of 4 weeks and the cells were inspected at day 1 and after 2 and 4 weeks [3].

2.4.3 Method 3: After RPE cells spontaneously detached from the flat mounts and continued to grow on the plastic

In this case, the eyecup was also left entire, as in method 2 and just small incisions were made at the marginal area to get flat eyecups. For the following studies the eyecups were put into a 35 x 10mm cell culture dish (Greiner Bio-One GmbH, Frickenhausen, Germany) with 3-4 ml DMEM-medium and they were investigated by phase-contrast microscopy every second day. The medium was changed every third day. At day 1 and after 2, 4 and 8 weeks the eyecups were removed from the culture dishes and the cells that settled on the plastic bottom were counted. The cells were additionally subcultured after 2 weeks and 4 weeks in 24-well plates. Furthermore, the remaining eyecups were observed under a light microscope [3].

2.5 Quantification of cells

2.5.1 Cell viability

Trypan Blue staining was performed to determine the cell viability. Trypan Blue is a stain that is actively extruded from viable cells, but which readily enters and stains dead cells. Therefore, the cells which are blue are dead. The difference between the total number of cells and the number of dead cells is the number of viable cells in a given aliquot of the culture.

A suspension culture was gently swirled to distribute the cells evenly. Then we aseptically removed a small sample (0.1 ml) of cells from the cultures and placed it in a separate test tube. 0.1 ml of the 4:1 diluted dye was added to the sample and everything was gently mixed. We then immediately placed a drop of the stain/

culture combination on the stain counting chamber and waited one minute. We observed the cells by light microscopy and counted the total number of cells. Then we computed the concentration of viable cells per milliliter of culture.

2.5.1.1 Procedure for freshly isolated cells

A suspension culture was gently swirled to distribute the cells evenly. Then a small sample (0.1 ml) of cells from the cultures was aseptically removed and the sample was placed in a separate test tube.

Four parts of stock Trypan Blue were diluted with 1 part of 5 x saline. 0.1 ml of the diluted dye was added to the sample and everything was gently mixed.

A cell counting chamber and cover slip was set up. A drop of the stain/ culture combination was immediately placed on the cell counting chamber and it was waited one minute. The cells were observed with low power microscopy, the total number of cells and the number of stained cells was counted.

The concentration of viable cells per ml of culture was computed then.

2.5.1.2 Procedure for dead cells in the supernatant of a cell culture

The supernatant medium of a culture well was collected which may contain dead cells. The cells were washed, span down in PBS and it was continued as described above under point 2.5.1.1.

2.5.1.3 Procedure for dead cells that are adherent to a well plate

4 parts of 0.2% Trypan Blue were diluted with 1 part of saline and the diluted dye was added to the adherent cells. The dye was washed out with PBS and blue cells were counted under the microscope or in light micrographs.

2.5.2 Cell counting

To count the RPE cells of methods 2 and 3, a Neubauer chamber was used. For method 1 the number of cells was determined by evaluating several sections of the flat mounts under a phase contrast microscope.

The coverslip was placed over the counting surface prior to putting on the cell suspension. The suspension was introduced into one of the V-shaped wells with a pipet, then the area under the coverslip filled by capillary action. The charged counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power. Four squares were counted, the mean value computed and the values were converted into the whole cell suspension.

2.6 Light and electron microscopy

Confluent cell cultures, flat mounts and the neural retinas were fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Specimens were postfixed with 1% OsO_4 and at room temperature in 0.1 M cacodylate buffer (pH 7.4) for three hours, en bloc-stained with uranyl acetate and lead citrate and embedded in Epon after dehydration in a graded series of acetones. Cells were removed from the well plate using a jeweller`s saw and semi- or ultrathin sections made together with the plastic bottom of the well. Semithin sections were examined by light microscopy (Zeiss Axioplan2 imaging®, Zeiss, Jena, Germany). For electron microscopy, the sections were cut ultra - thin, post - stained with lead citrate and analyzed with a Zeiss 902 An electron microscope (Zeiss, Jena, Germany) [3].

2.7 Immunocytochemistry

To evaluate the quality of the cultured RPE cells from each method we focused on the tight junctions which separate proteins localized in the apical and basal part of intact RPE cells.

Therefore, the medium was removed and the cultures and flat mounts were washed twice with PBS. The cells were fixed in 4% paraformaldehyde at room temperature for 10 minutes and subsequently washed again in PBS three times at 10 minutes each. Then they were permeabilized with 0.25% Triton X-100 in

PBS for 10 minutes and rinsed for a third time in PBS. Rat monoclonal Anti-ZO-1 (Santa Cruz Biotechnology, USA, CA) was used at a dilution of 1:400 in PBS buffer [38]. The primary antibody was incubated overnight at 4°C. The next day the cultures were washed twice in PBS for 10 minutes each prior to incubation with the secondary antibody. The cells were then incubated at room temperature for 30 minutes with a Cy3 conjugated goat anti-rat IgG (H+L) (Jackson Immuno Research inc. provided by DIANOVA Hamburg, Germany) diluted to 1:400. All antibodies were diluted in diluents (S2022) from Dako (Glostrup, Denmark). Then the cells were washed three times in PBS at room temperature for 5 minutes. Cells were covered with fluorescent mounting medium (Dako, Glostrup, Denmark) and inspected with a fluorescence microscope 510; (Carl Zeiss, Oberkochen, Germany) [3].

2.8 Effect of the test substance

Passage 0 or passage 1 cells from SD rats were used for all experiments. The test substance was used at a concentration of 3 μM. The test substance was added to the medium two days after the cell were seeded and the cells were cultivated two weeks in the presence of the test substance. Medium without the test substance was served as control and the medium was changed twice a week.

The effect of the test substance was determined on five chosen endpoints: Cell viability, phagocytic activity, the effect of repeated ROS feeding- hypertrophy, cell polarity and protein synthesis.

2.8.1 Cell viability

By Trypan Blue staining was performed to determine the viability after two weeks of culture in the presence or without 3 μM of the test substance. The procedure of Trypan Blue staining is described in 2.5.1.

2.8.2 Phagocytic activity

For the feeding experiments, isolated rod outer segments (ROS) from pigs were used because it is known that the amount of ROS uptake is not influenced by the donor species. Isolated porcine retinae were agitated for 2 min in buffer containing KCL (0.3 M), Hepes (10 mM), $CaCl_2$ (0.5 mM), $MgCl_2$ (1 mM) and sucrose (48%) at pH 7.0. The suspension was centrifugated at 7000 rpm (ALC centrifugette 4214, ALC International, Milan, Italy) for 5 minutes then the supernatand is filtered through a column filled with gauze, diluted with KCL buffer (1:1) and centrifugated again at 5000 rpm for 7 minutes. The isolated ROS were stored at 20°C until use.

Flow cytometry was used to quantify rod outer segment phagocytosis in cultured retinal pigment epithelial cells from Sprague Dawley rats after 2 weeks in culture. RPE cells were grown on 24-well plates in which they reached confluence. For the phagocytosis assay, ROS were labeled with 10 mg/ml FITC (following an established method from McLaren et al, 1993) [39] pelleted and rinsed 4 times in phosphate buffered saline (PBS). RPE cells were challenged with FITC-ROS for 15 hours (200 µl of ROS suspension in abundance per well). Afterwards, they were rinsed 3 times with PBS, trypsinized and prepared as a single cell suspension. The necessity to use trypsin on cells in order to create a single cell suspension results in the complete removal of all surface-bound ROS. The cells were counted and at least 10^5 cells in 100 ml were used for each run. The fluorescence of 10000 events from 100 000 cells was assayed on a Becton Dickinson LSR II flow cytometer using a live gate to exclude cells fragments, ROS particles, and other unwanted debris.

2.8.3 Effect of repeated ROS feeding - hypertrophy

For each experiment RPE cells that had been adapted to the culture conditions and had reached confluency were exposed to ROS suspended in 150 µl complete medium at 37°C. In every case the cells were completely covered with ROS. After 4 h the rod fragments that had not been phagocytized were washed off with PBS. The cells were challenged with ROS this way seven times during 2 weeks.

2.8.3.1 Quantification of lipofuscin-like autofluorescence

Lipofuscin - like autofluorescence was quantified after two weeks of culture by using a BIO-TEK Synergy HT plate reader (BIO-TEK instruments, Vermont, USA) with 360/40 – 528/20 filters.

The cells were divided into four groups:

- Group A: Cells without the test substance and without ROS
- Group B: Cells without the test substance and with ROS
- Group C: Cells with the test substance and without ROS
- Group D: Cells with the test substance and with ROS

2.8.3.2 Electron microscopy

The same four groups described in 2.8.3.1 were investigated under electron microscope and the cell thickness was measured with ITEM analysis software (Olympus). The results were obtained after three weeks culture. Therefore, confluent cells were fixed and prepared for electron microscopy as described in 2.6.

2.8.4 Cell polarity

The cell polarity was investigated by electron microscopy and was judged by location of apical microvilli, lateral tight junctions and basal labyrinth.

Tight junctions

As a marker for the evaluation of cell polarity, ZO-1 (Zonula Occludens-1) was used, as described in 3.7. *The expression of ZO-1 is determined as a marker for the formation of epithelial tight junctions. These junctions separate proteins localized in the apical and basal part of RPE cells. Cellular location of the protein in healthy cells is shown as a hexagonal shape on the cell membranes* [3].

2.8.5 Protein synthesis

AHA Alexa Fluor 488 Protein Synthesis HCS Assay (invitrogen; Paisley, UK) was used. The assay provides a fast, sensitive, non-toxic, and non-radioactive method for the detection of nascent protein synthesis utilizing fluorescence microscopy and high throughput imaging. AHA (L-azidohomoalanine) is an amino

acid analog of methionine containing an azide moiety. Similar to ^{35}S-methionine, AHA is added to cultured cells and the amino acid is incorporated into proteins during active protein synthesis. Detection of the incorporated amino acid utilizes a chemoselective ligation or click reaction between an azide and alkyne, where the azido-modified protein is detected with an Alexa Fluor 488 alkyne.

1. The cells were placed at desired density in black 96-well plates. (Nunclon^{TM}Surface,Nunc, Roskilde, Denmark)
2. After 2 days cells were treated with the test substance for 2 weeks.
3. A working stock solution of AHA (Component A) was prepared by diluting 1:1,000 in prewarmed L-methionine-free medium for a 50 µM final working solution.
4. Drug - containing medium was removed and 100 µl/well of medium with 50 µM AHA working solution were added.
5. Incubation for 30 minutes under conditions optimal for the cell type.
6. After incubation, the medium containing AHA was removed and the cell
7. were washed once with PBS.
8. 100 µl/well 3.7% formaldehyde were added in PBS, incubated for 15 minutes at room temperature and then the fixative was removed.
9. Cells were washed twice with 3% BSA in PBS and the wash solution then removed.
10. 100 µl/well 0.5% Triton X-100 were added in PBS and incubated for 20 minutes at room temperature.
11. 1 x AHA buffer additive was prepared by diluting the 10 x solution 1:10 in deionized water. The preparation of this solution must be fresh and used on the same day.
12. The reaction cocktail was prepared and used within 15 minutes of preparation.
13. The permeabilization buffer was removed and the cells were washed twice with 100 µl/well 3% BSA in PBS. The wash solution was removed.
14. 100 µl/well reaction cocktail was added to each well and well mixed.

15. Then it was incubated for 30 minutes at room temperature, protected from light.
16. Each well was washed twice with PBS the wash solution was removed.
17. The reaction cocktail was removed and the wells were washed once with 3% BSA in PBS.
18. PBS was added to each well. The plate was sealed with plate sealing film, if desired.
19. The plate was scanned by using automated imaging platform BIO-Tek Synergy HT (BIO-TEK instruments, Vermont, USA) with 485/20 and 528/20 filters appropriate for FITC. Nascent protein synthesis was assessed by determining signal intensity in the FITC channel.

Chemicals and antibodies	***Manufacturer***	***Lot/ Ref.-Number***	***Characteristics***
DMEM-Medium (Dulbeceo's modified Eagles' medium)	*GIBCO*	*31885-023*	*+ 1 g/L D- Glucose* *+ L-Glutamine* *+ Pyruvate* *+ 10% FCS (fetal calf serum)* *+ 1% Pen Strep*
FCS/FBS *(Fetal Bovine Serum)*	*GIBCO*	*41F0693K/10270-106*	
Pen Strep (Penicillin Streptomycin)	*GIBCO*	*708421/15140-122*	
PBS (-)(Phosphat Buffered Saline, Sigma)	*GIBCO*	*758132/14190-94*	*- Calcium* *- Magnesium*
PBS (+)(Phosphat Buffered Saline, Sigma)	*GIBCO*	*745388/14040-091*	*+ Calcium* *+ Magnesium*
Trypsin	*GIBCO*	*76301425200-072*	*0,25 % Trypsin-EDTA*
Trypan blue	*GIBCO*	*1190600/15250-061*	
Rat monoclonal anti-ZO1 antibody	*Santa Cruz Biotechnology*	*F1609*	*Dilution: 1:400*
Cy3 labeled goat anti-rat antibody	*Jackson Immuno Research*	*76950*	*Dilution: 1:400*
PFA (paraformaledhyd)	*Merck*	*1.04005.1000*	
Triton X-100 Solution	*Fluka (Biochemika)*	*1344815*	
Diluent (Antibody Diluent)	*Dako (Dako REALTM)*	*00057069/S2022*	
Ethanol			*70%*
Fluorescent mounting medium	*DAKO*	*S302380-2*	

Table 1

Used Chemicals and antibodies [3]

III. Results

3.1 Retinal Adhesion

Artificial retinal detachment can be performed easily and rapidly, directly after dissection, but not without causing cellular damage. In particular, the removal of the retina from freshly enucleated eyes of adult rats is critical, because the retinal pigment epithelium adheres firmly to both the choroid and retina. In 6-10 day - old rat retinal adhesion does not feature so strongly because their eyes have little outer segment material.
As Fig. 4 a from a pigmented Long Evans Rat shows, there are black spots adhering to the retina after removal without any other pre-treatment. [3].

Figure 4 a
A freshly removed retina, without any pre-treatment, from a pigmented Long Evans rat shows there are black spots adhering to the retina (ellipse). These black spots are numerous microvilli that were wrenched off the flat mounts during the preparation process. The arrow marks the position where the optic nerve was cut out. The maximum density of the black spots is located at the peripheral position of the retina (magnification 10x) [3].

These black spots consisted of apical parts of the RPE with microvilli and melanosomes (Fig. 4b) as electron microscopy shows (Fig.4c).

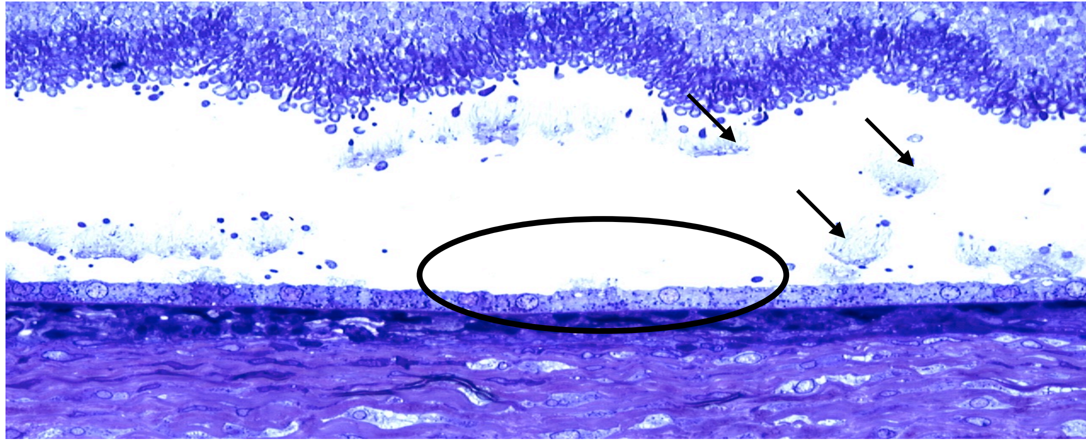


Figure 4 b

A semi-thin section shows the retina with detached microvilli that were torn off the RPE cells when no further treatment was carried out before preparation (arrows). Such microvilli are shown at the ultrastructural level in Fig 4 c. The ellipse marks a section of RPE cells without any microvilli. (magnification 40x) [3].

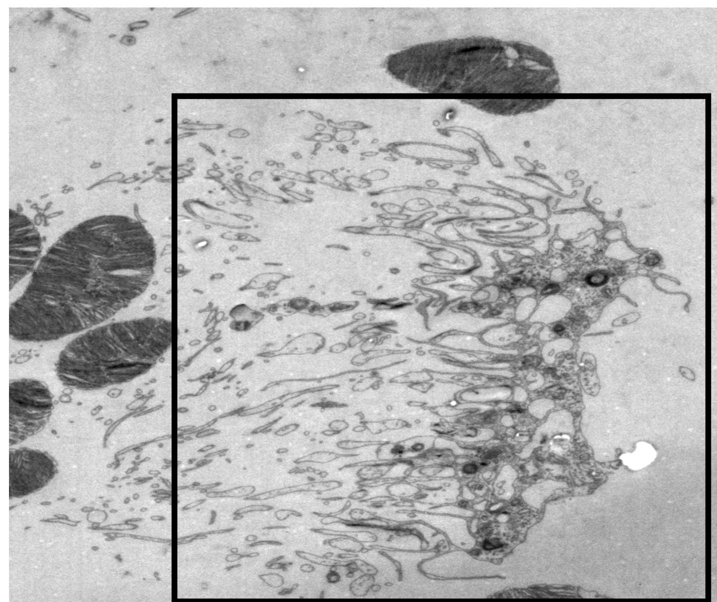


Figure 4 c

An electron micrograph shows the isolated apical part of a RPE cell with microvilli after removing the retina.(black box) (magnification 4400x) [3].

With this approach, the yield of viable RPE cells was very low. To overcome the retinal adhesion, it was most successful to soak the eyes overnight in DMEM at 4°C before removing the retina, as described in 2.3, approach 3. A semi-thin section (Fig. 4b) shows the retina with detached microvilli which were torn off the RPE cells when no further treatment was carried out before preparation. In comparison figure 4 d shows that the RPE cells separate complete with microvilli, but they form evaginations [3].

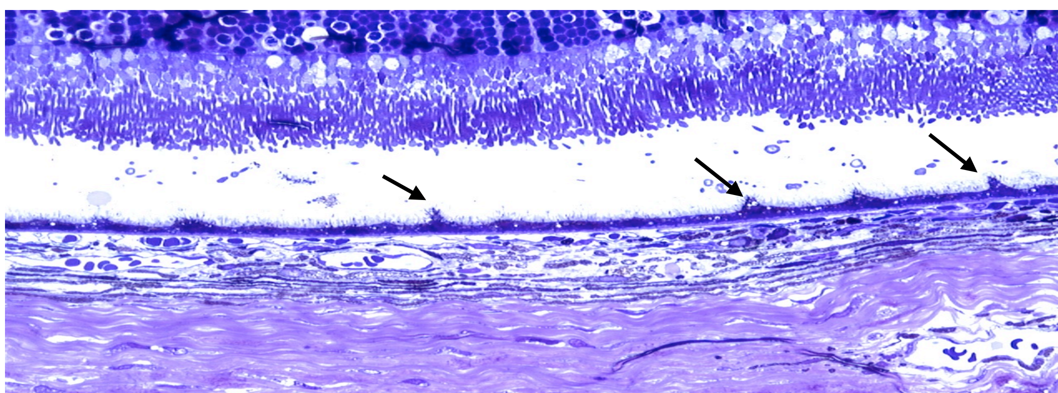


Figure 4 d

In comparison, after soaking the eyes overnight in DMEM at 4°C, the semi-thin section shows that the microvilli stay on the RPE cells, but they form evaginations (arrows). (magnification 40x) [3]

The pre-treatment with enzyme solutions in different concentrations for varying times did not yield further improvements. But after incubating the eyes overnight, to give the RPE cells the possibility to disassociate their microvilli from the neural retina, the black spots adhering to the retina were greatly reduced [3].

3.2 Morphology

3.2.1 Method 1

Figures 5 a - d show the RPE cells on the flat mounts from pigmented and albino rats. In some flat mounts the cells are completely absent, in other sections they are readily identifiable with a hexagonal shape. Figure 5 a shows some dead cells with dark nuclei stained by Trypan blue as well as some living cells. Figure 5 b shows an overview of a flat mount from an albino rat fixed directly after preparation. Figures 5 c & d show a closer view of the recognizable RPE cells

from a pigmented rat directly after enucleation (Fig 5 c) and from an albino rat after 1 week in culture (Fig 5d). Directly after preparation (Fig 5 c), the cells are densely packed and show in vivo morphology, but after 1 week in culture (Fig 5 d), they detach from each other [3].

Fig. 5 a-d: A sample of bright - field light micrographs from flat mounts of method 1.

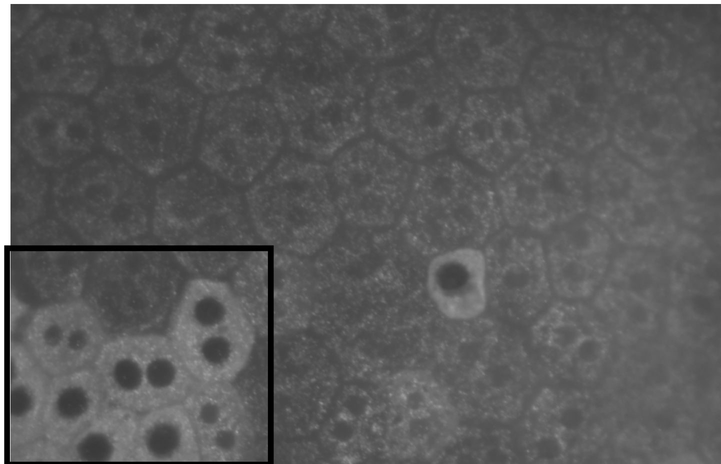


Figure 5 a

The trypan blue staining performed on flat mounts from pigmented rats. On the bottom left of the Figure (box), there is a group of cells which absorbed the dye indicating that these cells are dead (magnification 400x). [3]

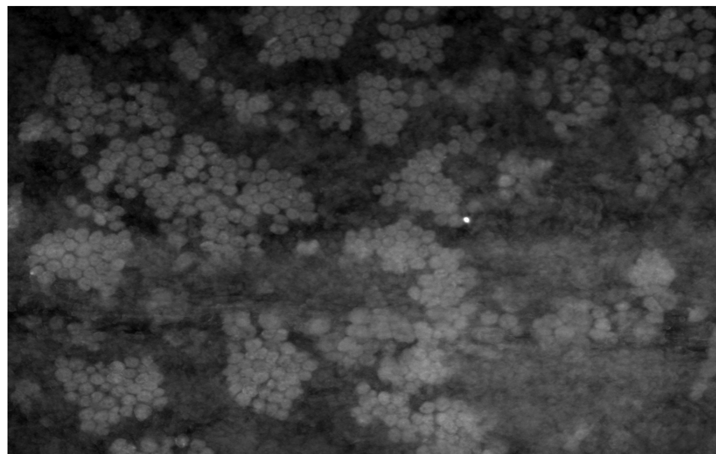
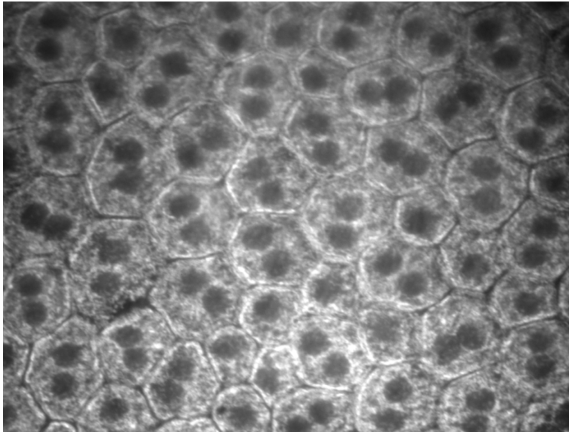


Figure 5 b

An overview of an albino flat mount directly after preparation shows that the cells are not presented all over the tissue (magnification 100x). [3]



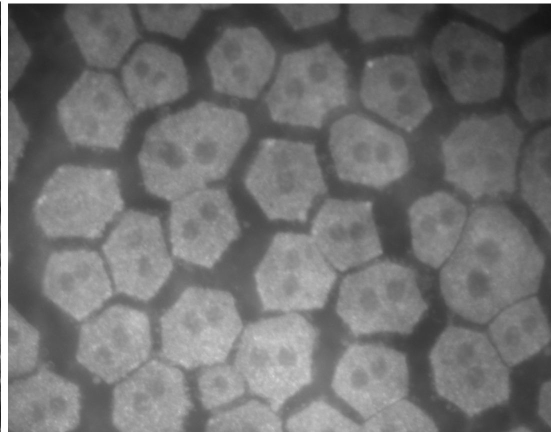


Figure 5 c **Figure 5 d**

The flats show a closer view of the discernible RPE cells from pigmented (c) and albino (d) rats. The density of the pigmented cells corresponds to the early point of time, when the cells are not yet separated. After one week of culture (d) the cells drifted apart (magnification 400x). [3]

3.2.2 Method 2

The "conventional" cell culture comprises clearly recognizable RPE cells which change their original morphology from hexagonal cells to rather oval cells. Such a result from pigmented rats after one week of culture is shown in figure 6. The pigmentation is retained and confluence is nearly achieved [3].

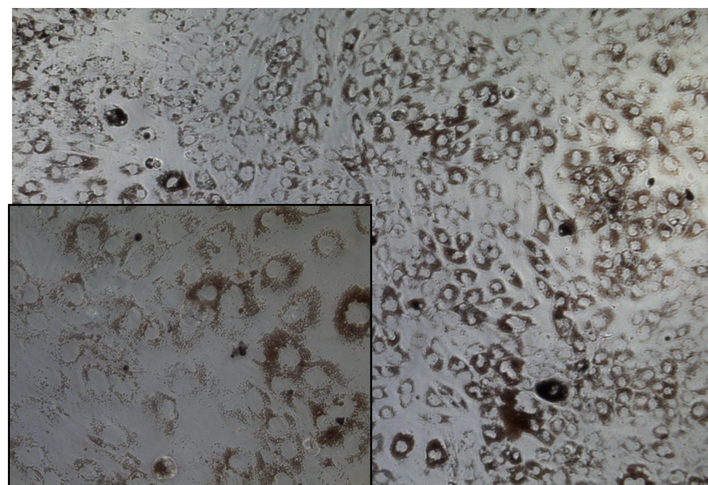


Figure 6

Light micrographs of RPE cells of Long Evans rats plated in a 96-well chamber after one week (magnification 100x / insert 400x). [3]

3.2.3 Method 3

On the flat mounts the RPE cells migrated off the tissue and settled on the plastic bottom of the petri dish (Fig 7a).

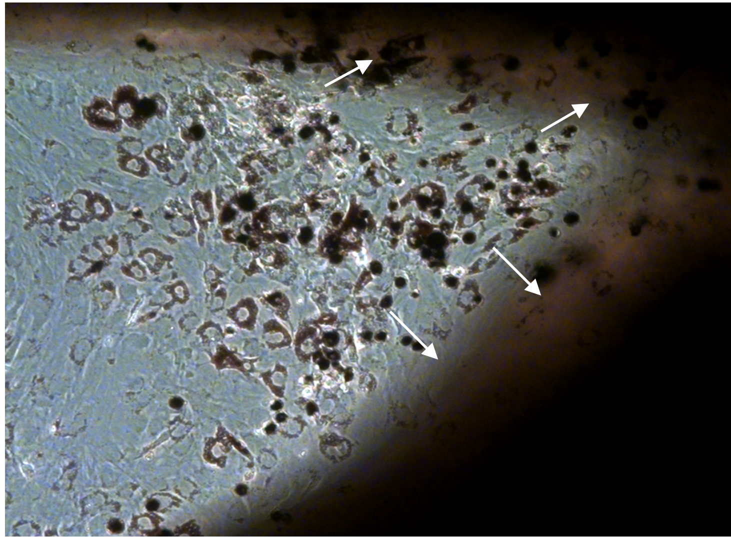


Figure 7 a

The cells from method 3 drift off the tissue and settle on the plastic bottom of the petri dish between an incision into the flat mount (dark area). The white arrows mark the border of the flat mount, where the cells came off. Shown is an example of a pigmented rat. (magnification 100x) [3]

The morphology of the cells, when growing on the plastic, is similar to the cells from method 2. As shown in both other methods, tight junctions are also present as seen in electron microscopy [3] (Fig 7 b). The greatest cell density was found at the border of the flatmount. The pigmentation of Long Evans rats was retained, as seen in figure 7 a. Confluence is reached within 7- 10 days.

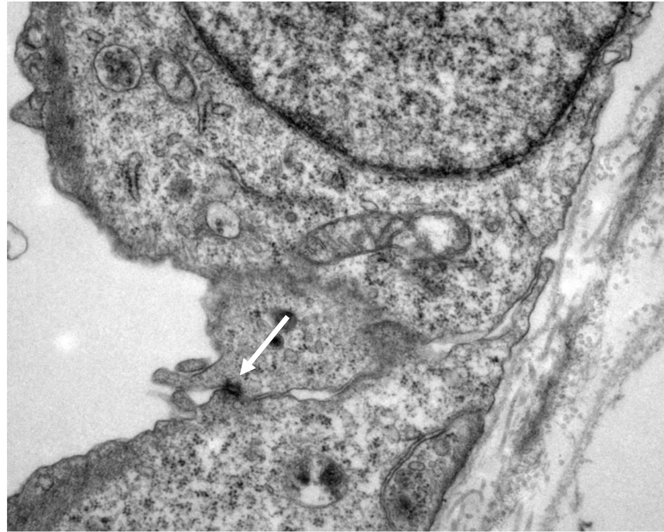


Figure 7 b

An electron micrograph from method 3 shows the cell border of two RPE cells connected by tight junctions (white arrow). (magnification 20000x) [3]

3.3 Yield

3.3.1. Method 1

The yield from the flat mounts, fixed directly after preparation is up to 30 000 ± 6900 cells/eye. After 2 weeks in culture there are 18 000 ± 6200 cells/eye and after 4 weeks in culture 11 000 ± 5800 cells/eye [3]. Figure 8 shows the number of cells of all three methods at day 1 and after 2 and 4 weeks. The highest cell number and growth rate gives method 3. In method 1 the cells that drift off the tissue were not counted. *In method 3, the number of cells settled down on the flat mounts surface at day 1 is indicated, but after 2 and 4 weeks only the cells growing on the surface of the petri dish are quantified* [3].

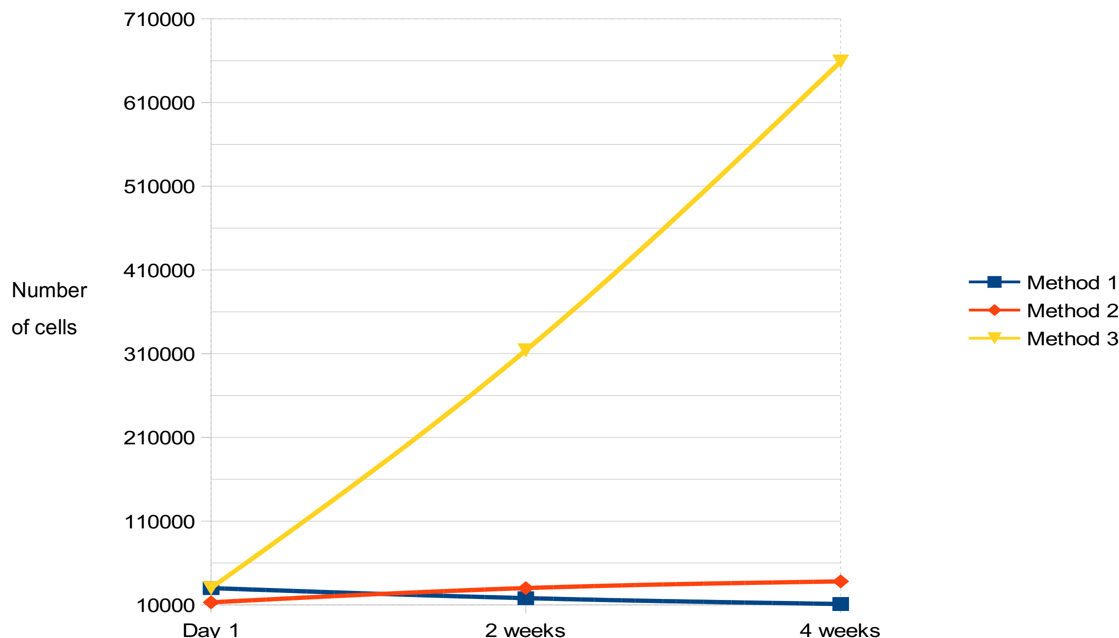


Figure 8

The graph shows the average cell yield of all three methods at day 1 and after 2 and 4 weeks. It is clearly recognizable that method 3 gives the highest cell numbers and a high growth rate. In method 1 only cells on the surface of the flat mounts are counted. In method 2 cells are always in the petri dish. In method 3, the number of cells settled down on the flat mounts surface at day 1 is indicated, but after 2 and 4 weeks only the cells growing on the surface of the petri dish are quantified. [3]

3.3.2 Method 2

RPE cells from pigmented rats grow to confluence within 1 week, using a cell density of 15 000 – 20 000 cells/well. The yield of an individual eye is about 13 000 ± 6300 RPE cells directly after preparation (day 1), including 3-4% dead cells. After 2 weeks, the yield was 30 000 ± 6400 cells/eye and after 4 weeks, 38 000 ± 6800 cells/eye (Fig 8) [3].

3.3.3 Method 3

We cultivated the cells in 35 x 10 mm cell culture dishes and counted them at different points of time. In contrast to method 1 and 2, we additionally subcultured the cells. The first test series ran 2 weeks, 4 weeks and 8 weeks.

Within 7-10 days we reach confluence with a cell number of 180 000 cells per well. In this case the number of cells directly after preparation was the same as in method 1 (30 000 cells/ eye). After 2 weeks the cell count of the cells that settled on the plastic resulted in 314 000 ± 174 000 cells/ eye. After 4 weeks the number grew up to 659 000 ± 185 000 cells/eye. After 8 weeks in culture the number of cells waned to 403 000 ± 194 000. There-upon we subcultured the cells after 2 weeks and 4 weeks in other culture bottles and after another 3 weeks gained average numbers of 1 700 000 cells. So, compared to the second method, the yield was 10-fold greater than the yield of the conventional method or the cells on the flat mount (Fig 8) [3].

3.4 Cell survival

3.4.1 Method 1

The analysis of the Trypan blue test directly after preparation shows that 4% of the RPE cells on the flat mounts were dead (Fig 5 a). After two weeks in culture there were 4,5% of dead cells counted [3].

3.4.2 Method 2

As indicated by Trypan blue staining, after two weeks of culture, 3 % of the isolated cells were dead. The counting directly after preparation could not be performed, because the cells of method 2 and 3 need time to settle on the plastic bottom of the petri dish [3].

3.4.3 Method 3

Trypan blue staining indicated that, exactly as in method 2, 3% of the RPE cells were dead after 2 weeks of culture [3].

3.5 Differences between pigmented and albino RPE cells

The use of both, the pigmented and albino rat cells in comparison, showed that they behave similarly in cell quantity and quality independent of the method used. By taking the example of method 2, figure 9 shows that the yield of living RPE cells per eye of albino (13 000 ± 8000 cells) and pigmented rats (13 000 ± 4700) was similar [3].

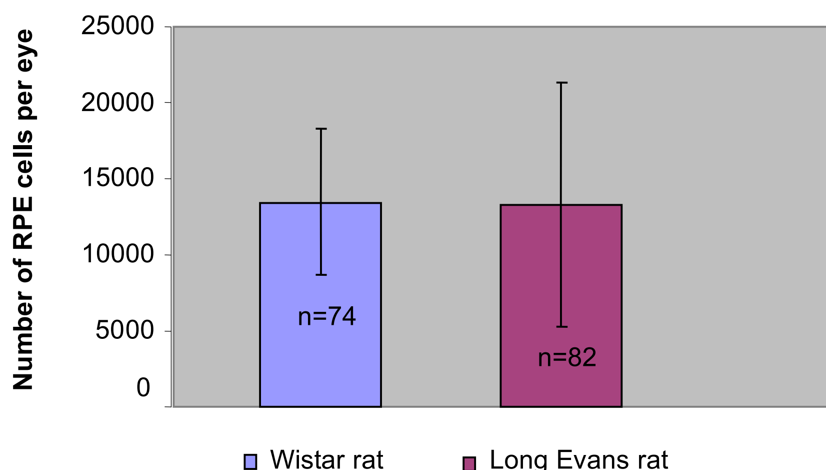


Figure 9

The graph shows the cells from method 2 counted directly after preparation. The yield of albino and pigmented rats was similar. 74 Wistar eyes yielded 13000 ± 8000 cells and 82 Long Evans eyes 13000± 4700 cells including 3-4% dead cells. [3]

3.6 Cell polarity

Cellular junctions and apical microvilli were present shortly after the cells had grown to confluence. A basal labyrinth was never seen in culture. With prolonged culture time cell polarity disappeared (Fig.10).

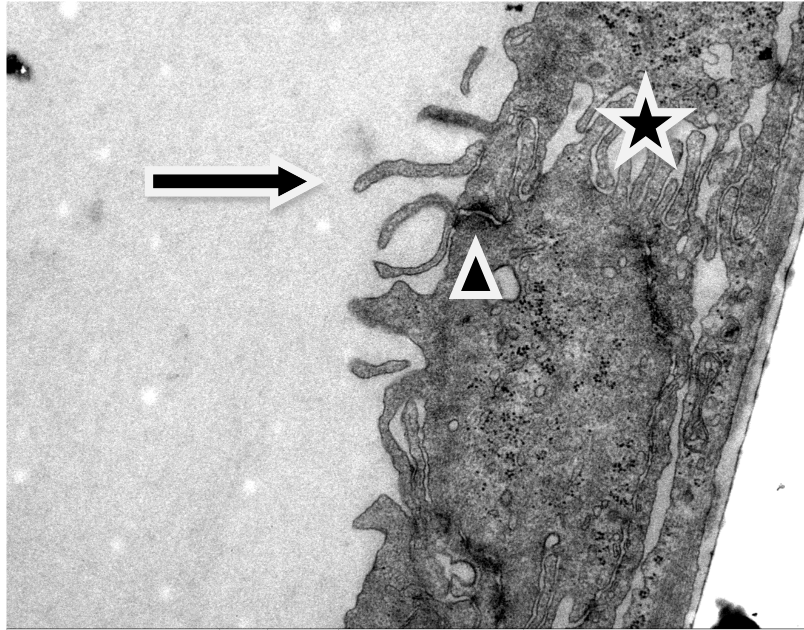


Figure 10
Cellular junctions (arrowhead) and apical microvilli (arrow) were present after 4 days of culture when the cells had grown to confluence. Within the desmosome (arrowhead) the tight junction is localized. A basal labyrinth is not present in culture. Adjacent cells are attached by interdigitations (asterisk).

One of the best ways to determine whether functional RPE cells have been successfully cultured is by staining for proteins integral to RPE function. ZO-1 (Zonula Occludens-1) is a protein that connects tight junction cytoskeleton. These junctions separate proteins localized in the apical and basal part of RPE cells. Cellular location of the protein in healthy cells is shown as a more or less hexagonal shape on the cell membranes (Fig 11 a) [3].

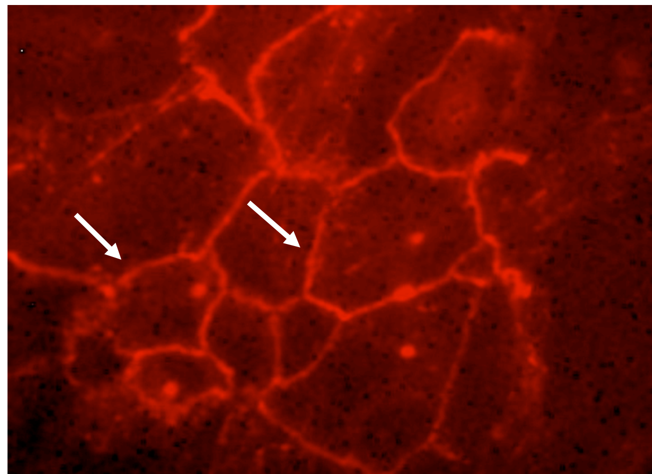


Figure 11 a

An example of the cells isolated from method 2 and 3 that grow on plastic for 2 weeks. Cells develop more or less hexagonal morphology and tight junctions (arrows). [3]

Cells cultured according to method 2 and 3 reached nearly hexagonal cobble stoned morphology and are connected by tight junctions (Fig 11b). [3]

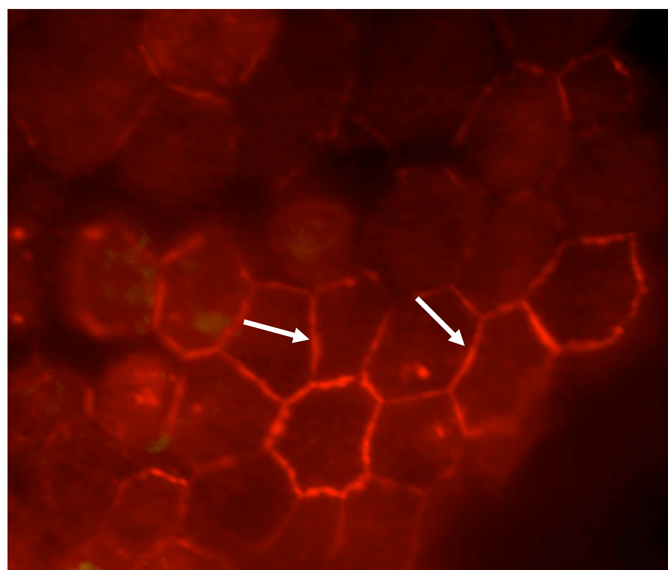


Figure 11 b

After two weeks in culture, the tight junctions from method 1 began to disappear and are only present between some cells (arrows) from a pigmented rat. [3]

The test substance from 2.8 did not influence the morphology of tight junctions or apical microvilli as judged by electron microscopy and immunocytochemistry.

3.7 Effect of the test substance

3.7.1 Cell viability

Cell viability was assessed by Trypan Blue staining. The RPE cells were cultivated in 96- well plates for two weeks (n = number of wells). No difference in the cell viability was observed after two weeks of culture in the presence or without 3 µM of the test substance (Fig 12.).

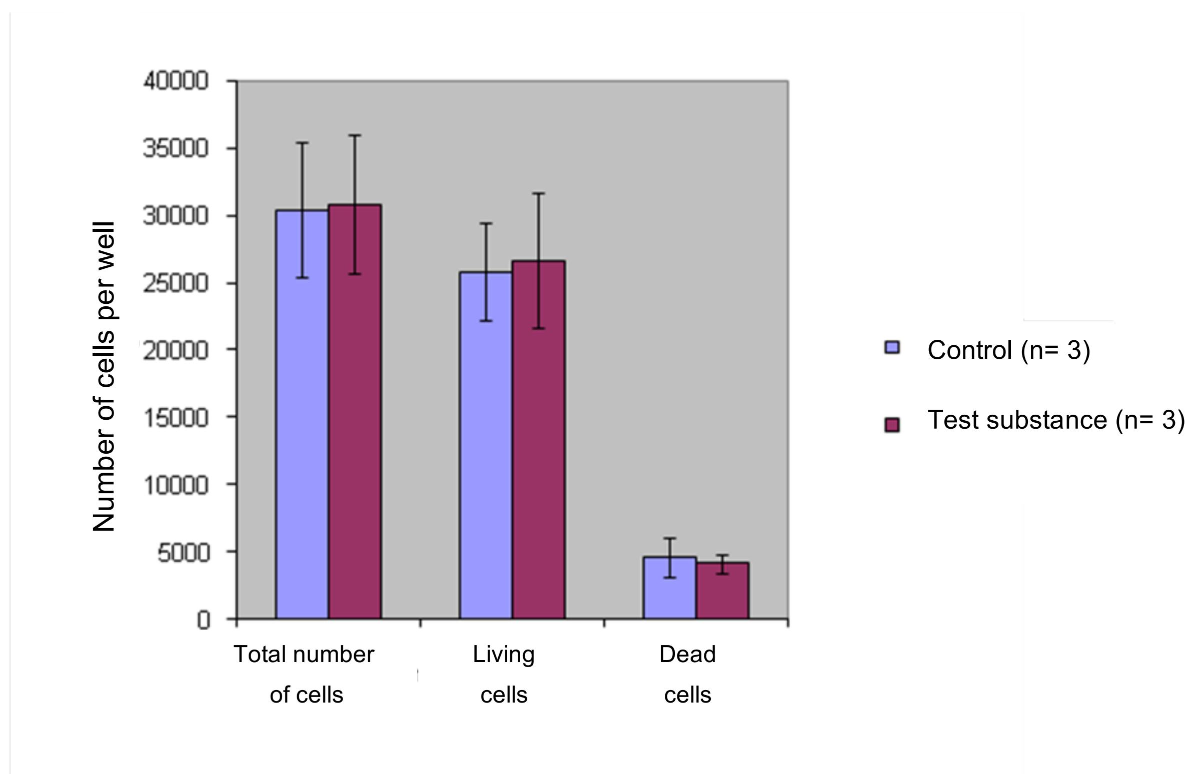


Figure 12

The graph shows the differences between the cell viability of culture in the presence or without the test substance.

3.7.2 Phagocytic activity

Group investigated	% of cells phagocytosing FITC-ROS	mean fluorescence intensity
Cells without the test substance and with FITC-ROS	86.05 ± 2.86 % (n = 4)	11288.5 ± 3917 (n = 4)
Cells with the test substance and with FITC-ROS	94.82 ± 0.54 % (n = 4)	9413 ± 630 (n = 4)

Table 2
Quantification of FITC-labeled rod outer segments (ROS) uptake by flow cytometry. Porcine ROS were used. The RPE cells were cultivated in 96-well plates (n = number of wells).

The percentage of cells phagocytosing FITC-ROS was significantly increased when the RPE cells were cultivated two weeks in the presence of 3 mM of the test substance compared to the cells cultivated without the test substance.
Concerning the mean fluorescence intensity, the difference observed between the two groups was not statistically significant. The mean fluorescence intensity indicates the level of phagocytosis. The higher it is, the higher the phagocytosis.

3.7.3 Effect of repeated ROS feedings- hypertrophy

The results of repeated ROS feeding are depicted in figure 13, by quantifying lipofuscin – autofluorescence after two weeks of culture.

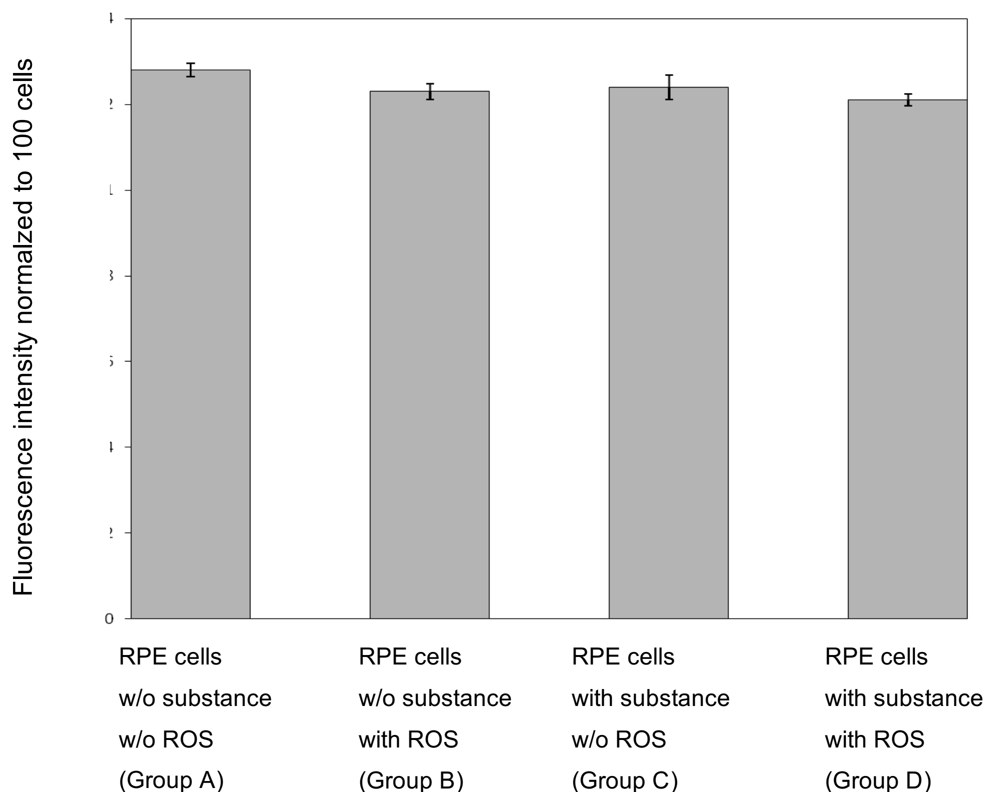


Figure 13

Quantification of lipofuscin-like autofluorescence normalized to the number of cells after two weeks culture. The RPE cells were cultivated in 96-well plates (n= 16 wells).

There was no significant difference in the lipofuscin - like autofluorescence between the four groups investigated. The treatment with 3 mM of the test substance and/or the repeated ROS feeding have not shown a distinct change in the lipofuscin content in the rat RPE cells after two weeks of culture, as shown in figure 13.

Electron microscopy

The main findings from RPE cells of SD rats after culture for 21 days under different conditions are shown in electron micrographs of figure 14:

- 14 a cell with culture in medium alone without treatment
- 14 b cells after culture in medium and after 10 feedings with ROS
- 14 c- f cells after treatment with 3 mM of the test substance
- 14 g after treatment with 3 mM of the test substance in combination with 10 feedings with ROS.

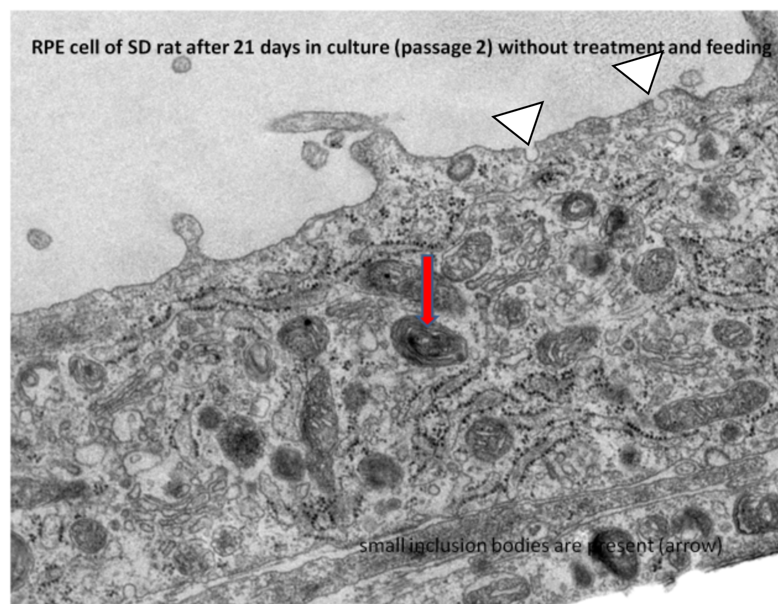


Figure 14 a

Without treatment, small inclusion bodies (red arrow) are present within the cytoplasm. At the apical cell border endo- or exocytotic vesicles (arrowheads) can be seen.

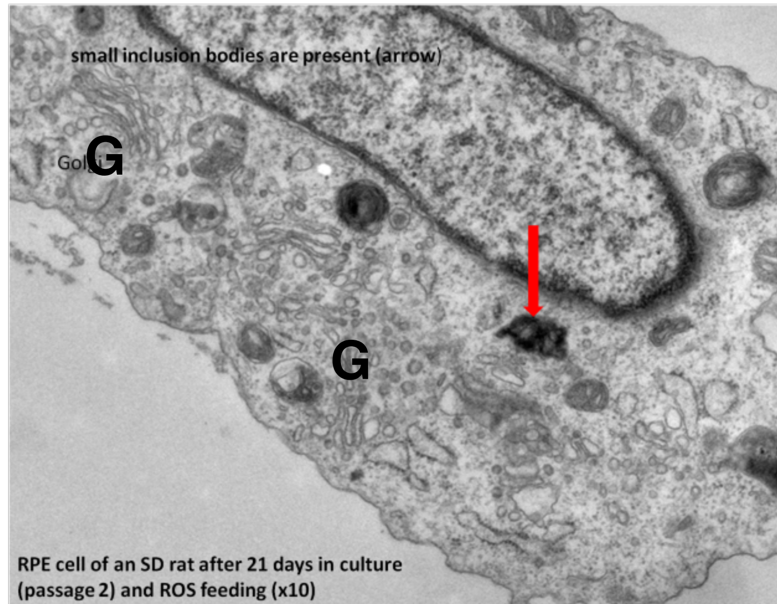


Figure 14 b

Without the test substance treatment but 10 ROS feedings small inclusion bodies (arrow) are present within the cytoplasm. Numerous Golgi bodies (G) are present.

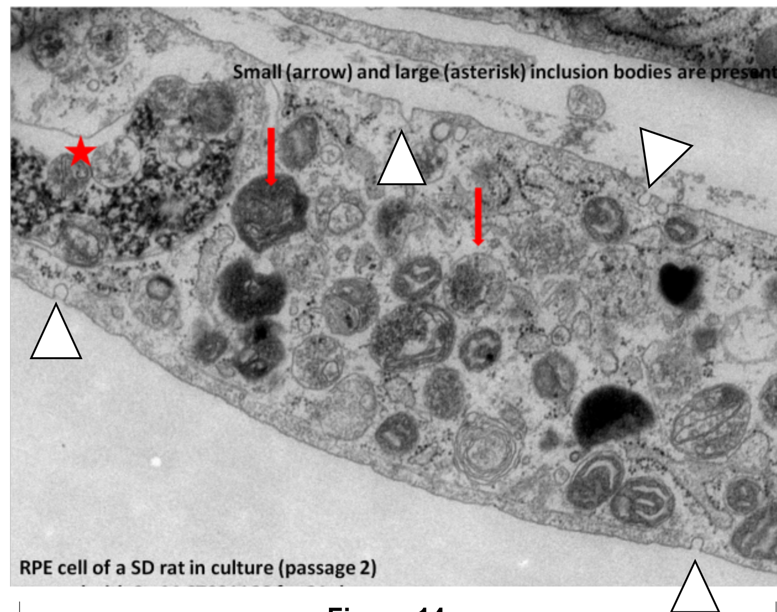


Figure 14 c

Treatment with the test substance alone enlarged the number of small inclusion bodies (arrows). In addition, a large inclusion body (asterisk) containing electron dense and electron opaque membrane bound material is formed. The latter may originate from mitochondria. At the apical and basal cell border endo- or exocytotic vesicles (arrowheads) can be seen.

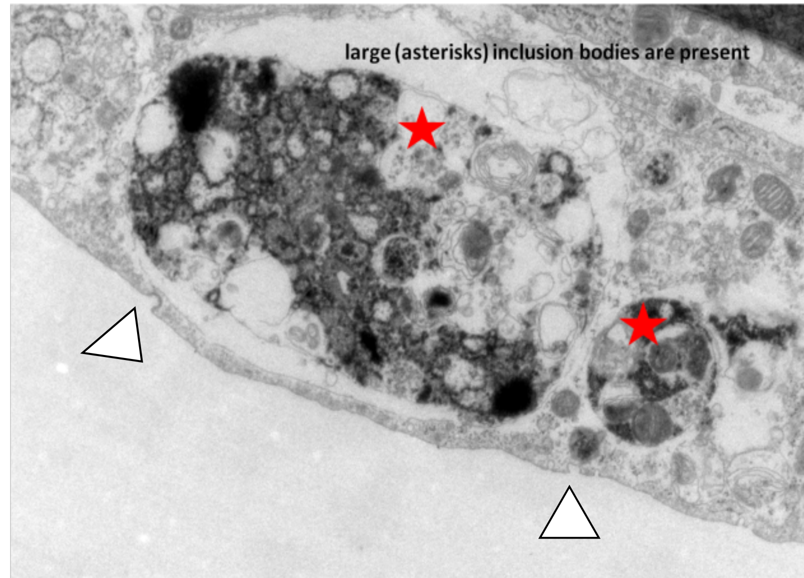


Figure 14 d

Treatment with the test substance alone induced the formation of large inclusion bodies (asterisks) containing electron dense and electron opaque membrane bound material is formed. At the apical cell border endo- or exocytotic vesicles (arrowheads) can be seen.

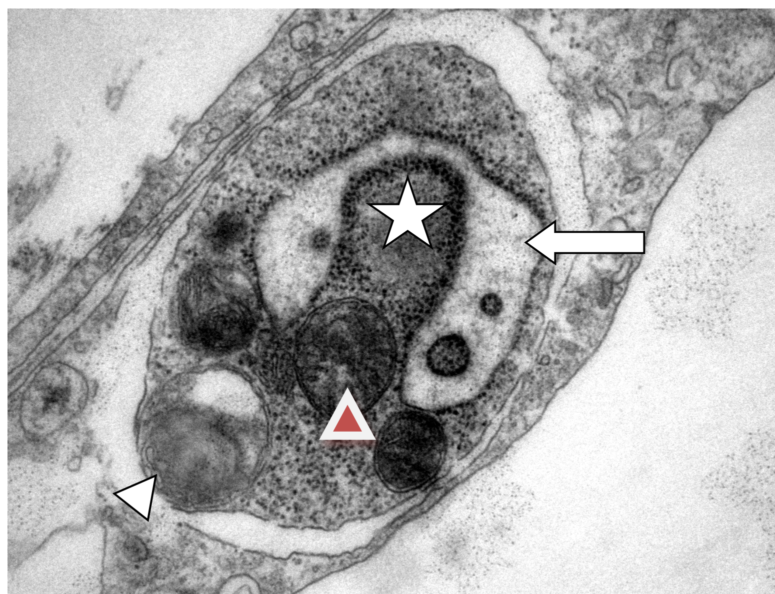


Figure 14 e

Treatment with the test substance alone induced the formation of a large inclusion body (asterisk) containing electron dense and electron opaque membrane bound material. One organelle inside this inclusion body can be identified as a mitochondrion by its double membrane (red arrowhead). The limiting membrane of the inclusion body is interrupted (white arrowhead). Rough endoplasmic reticulum (arrow) seems to be in train to invaginate the mitochondrium. All in all, this inclusion body appears to be an autophagosome.

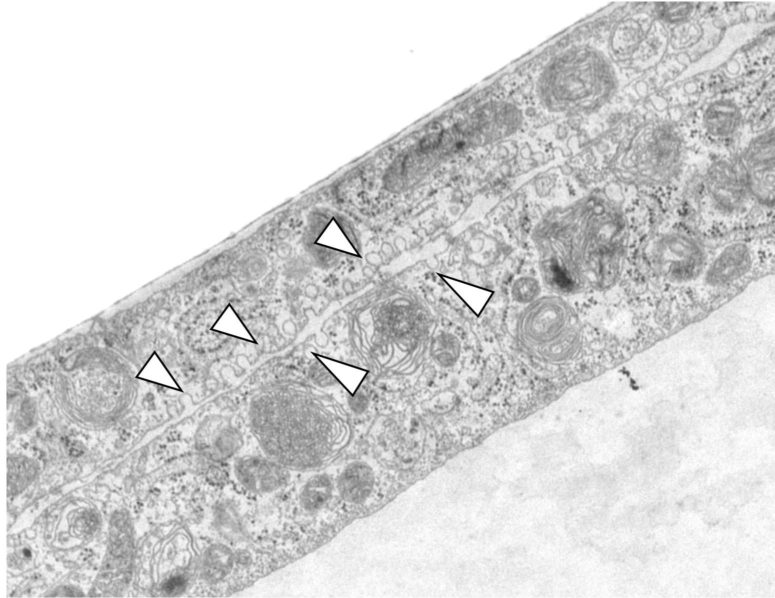


Figure 14 f

Treatment with the test substance alone induced the formation of many endo - or exocytotic vesicles (arrowheads). Here they are located between two adjacent RPE cells.

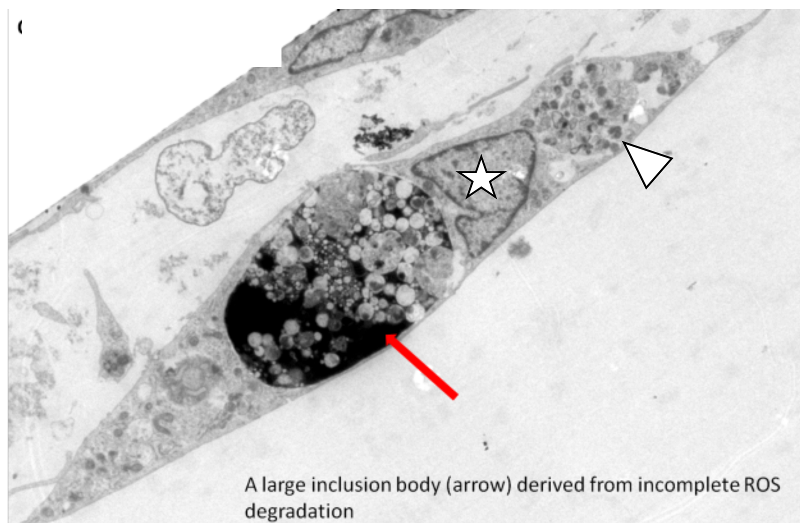


Figure 14 g

Treatment with the test substance in combination with repeated ROS feedings induced the formation of a very large (5 µm in length) inclusion body (arrow) containing electron dense and electron opaque membrane bound material in RPE cell of a SD rat. This was only seen in a few numbers of cells. After repeated feeding with ROS cells regularly detached from each other. This was also observed without the test substance treatment. The nucleus is labeled by an asterisk and a group of smaller inclusion bodies by an arrowhead.

Figure 15 a shows the RPE height in millimetre, comparing the four groups, described in 2.8.3.1.

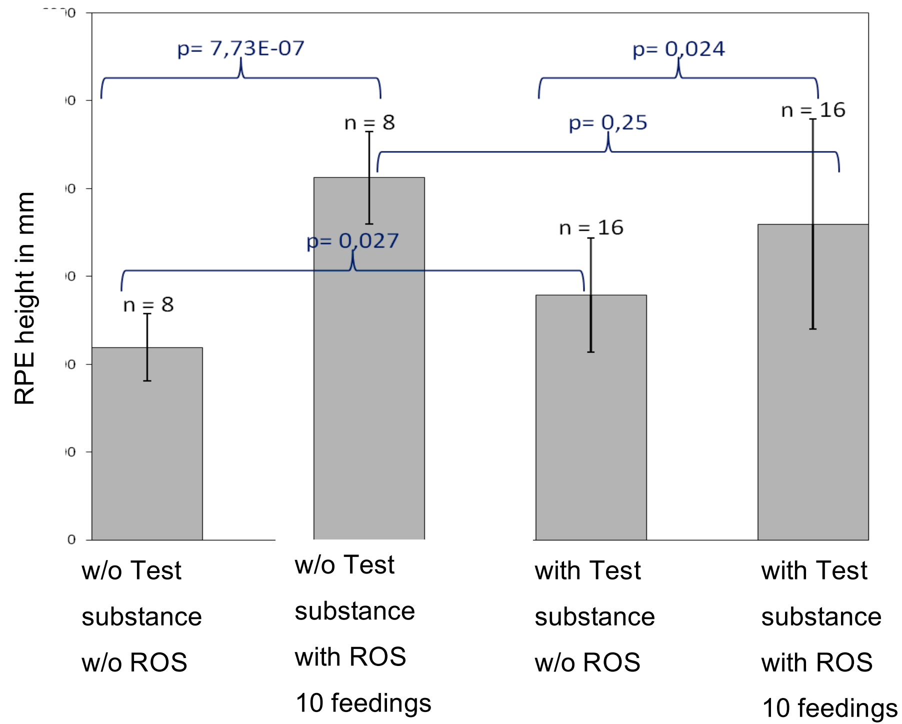


Figure 15 a
Measurement of the cell thickness in the four groups investigated.

The feeding with ROS alone enlarged the cell layer thickness significantly (Fig. 15 a). There was no significant difference of cell layer thickness between the cells that had been fed and treated with the test substance, compared with the cells that are fed only (Fig. 15 a).

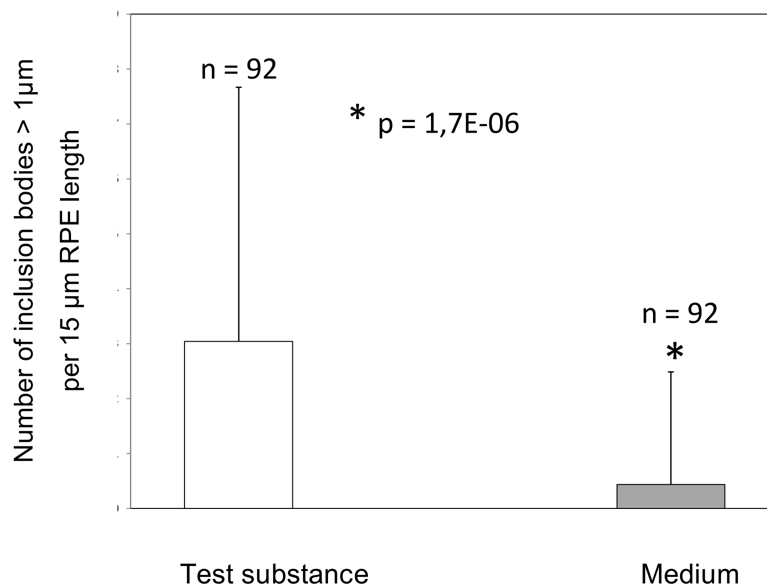


Figure 15 b
Measurement and counting of the number of inclusion bodies larger than 1 µm in diameter in 92 cells each group in electron micrographs. Treatment with the test substance enhanced the number of inclusion bodies.

However, individual cells accumulated large amounts of residual material after feeding and drug treatment (Fig. 14 b and 14 c). The treatment with the test substance alone enhanced significantly the cell thickness and induced accumulation of inclusion bodies (Fig. 14 d and 14 f). In figure 15 b it is clearly recognizable that the number of inclusion bodies larger than 1µm is enhanced when the cells were treated with the test substance.
Many cells following treatment with the test substance included electron dense vacuoles at a frequency of approximately every two to three cells observed. The described vacuoles were also observed in untreated cells though at a lesser frequency of approximately one out of every ten cells. There were also a few inclusion bodies observed without treatment which may be due to autophagy. However, this was not due to ROS feeding.

After repeated feeding with ROS cells regularly detached from each other, no matter of the test substance treatment.

3.7.4 Cell polarity

A basal labyrinth was never seen in culture. With prolonged culture time cell polarity disappeared.

The test substance did not influence the morphology of tight junctions or apical microvilli as judged by electron microscopy and immunocytochemistry.

3.7.5 Protein synthesis

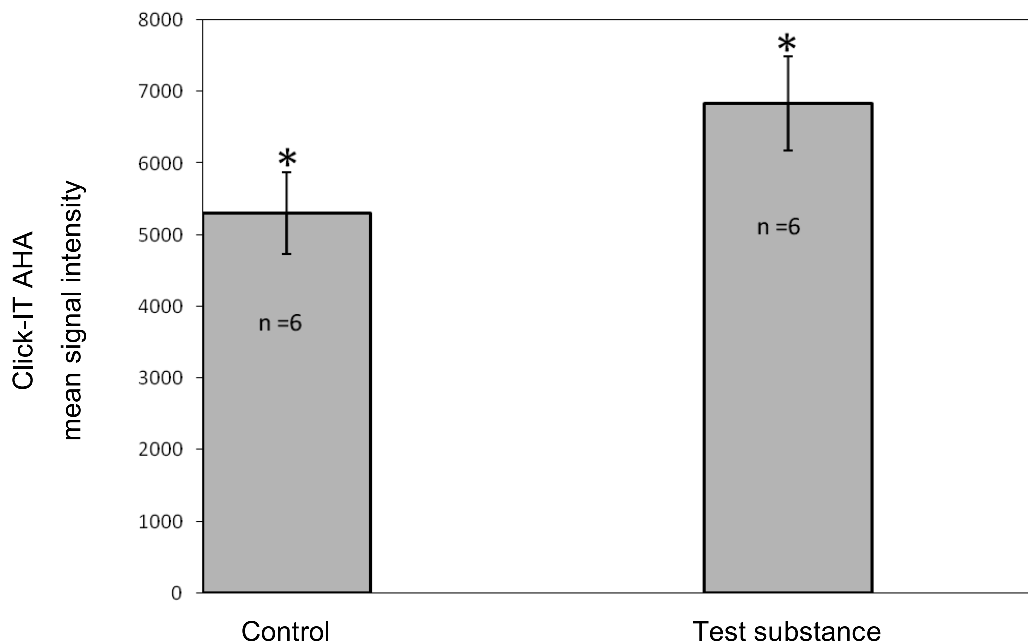


Figure 16

Protein synthesis assessed by the Click-IT AHA Alexa Fluor 488 Protein Synthesis HCS Assay (Invitrogen). The RPE cells were cultivated in black 96-well plates (n= number of wells).

The protein synthesis was statistically increased (*, $p = 0.0015$) after two weeks culture in the presence of 3 µM of the test substance (5302 ± 570) comparing to the control RPE cells that were not treated with the test substance (6837 ± 662). The percentage of inhibition of protein synthesis with 1 mM cycloheximide was low (maximal 15%). Anisomycin (1 mM) did not inhibit protein synthesis. The technical support from Invitrogen was informed but has no explanation.

IV. Discussion

In 1981, rat RPE cells were successfully isolated by Edwards [32]. He enucleated eyes from 6 – 8 day-old rats and counted 30000 – 60000 cells per eye. Shortly afterwards, Mayerson [33] published an improved method with 8 - 18 day-old rats by using hyaluronidase, collagenase and trypsin. He also collected RPE sheets with forceps and counted 29000 cells/ eye. His experiences with older rats provided very low yields. Both techniques do not permit RPE isolation from rats older than 10 postnatal days. For the first time, Wang [34] presented a method for adult rats. In 1995 Sakagami incubated the eyes in 0.1% proteinase K solution yielded an average of 4 x 10^4 viable RPE cells [40]. Therefore, in contrast to human [25]; [26]; [27], frog [31] or bovine RPE [28]; [29]; [30], there is no satisfactory method to isolate and culture adult rat RPE [3].

The aim of this study was to develop an improved method for isolating and culturing retinal pigment epithelial cells from adult rats, the most common laboratory animal. We presented three resembling methods whereby in the first method the RPE cells remain on Bruch´s membrane and in the second and third method the RPE cells grow on the plastic bottom of the culture dishes. The Bruch`s membrane, that is located between the RPE and the choroids plays a central role. On one side, it functions as a blood - retinal barrier and it also carries metabolic waste from the photoreceptors across the choroid.

4.1 Retinal adhesion

The main problem in isolating viable RPE cells is the adhesion to the retina and the risk of damaging the cells by separation from the outer segments.

The way to avoid this problem was the use of young animals at the age of 6-8 days as they do not have this strong retinal adhesion due to little outer segment material [3]; [32]; [33]; [41]. Additionally, the attempt was to treat the cells with different enzyme solutions before removing retina [33]; [34].

In our laboratory, our effort was to focus on adult rodents, wherefore we tried several protocols to compare the yield, preservation of morphology and viability. In general, adult cells are very fragile and can easily get damaged during the preparation process.

The reason for the strong adhesion between the RPE and the neural retina are numerous microvilli which are coated with an interphotoreceptor matrix [41]; [42]. Already at the age of 14-15 days the formation of photoreceptor outer segments begins to interdigitate with RPE apical microvilli [17]; [43].

Moreover, within the first two days of culture, the RPE cells secrete a fibrous mat of matrix materials from the basal surface [44].

Simple treatment with non-ionic detergent or mild trypsinization does not disrupt these adhesions, so the immediate removing of the retina after preparation is impracticable [32]; [45]. A suggestion from Mayerson was that rat RPE cells contain membrane proteins, or produce matrix components, which are insensitive or inaccessible to Trypsin [3]; [33]. Edwards [32] published first a protocol for isolating and culturing rat RPE from 6-8 day old rats. Before trypsinization he soaked the eyes 6-24 hours in BSS and yielded 30 000- 60 000 cells per eye. Another attempt for 8-15 day old rats was a pre-treatment with hyaluronidase and collagenase followed by trypsinization by Mayerson [33] yielding 29 200 ± 8 800 cells per eye. The same enzymes were used by Wang [34] for 4-14 day old rats. The most successful way to overcome the retinal adhesion in our laboratory was to soak the eyes overnight in DMEM at 4°C before removing the retina, as described in 2.3, approach 3. It is necessary to give the RPE cells the possibility to disassociate their microvilli from the rod outer segments of the neural retina. After the overnight incubation, the black spots adhering to the retina were greatly reduced.

4.2 Three Methods

We investigated three different methods and compared them with each other in quantity and quality. Methods 2 and 3 representing the culture with RPE settling on plastic and method 1 the culture with the RPE remaining on Bruch`s membrane, called flatmouns [3].

4.2.1 Method 1

This approach was to cultivate tissue flatmounts with the RPE attached on the sclera. One eye adduced 6 flatmounts with a size of 3 - 5 mm² (Fig. 3). We made observations by Light and Electron microscopy after different points of time (day 1 and after 2 and 4 weeks). This method is obviously the fastest and cheapest method of all. The preparation process is very simple, but nevertheless, the need of an overnight incubation is still required to overcome the interlinkage of the RPE microvill and rod outer segments. The next advantage is that the culture is a nearly pure culture, because the cells stay on Bruch`s membrane and the fibroblasts have no opportunity to grow. Additionally, the morphology of the cells is widely preserved after the dissecting process and the hexagonal shape is clearly visible, as shown by light microscopy.
The main problem with this method was that a lot of RPE cells were absent from Bruch`s membrane after 2 weeks. The survival of the cells was not foreseeable and depending on the dissection process, so the standard deviation was very high. *Figure 5 c shows a part of a flat mount from a pigmented rat, where the cells were well preserved and their hexagonal shape was definable. As shown by immunocytochemistry, RPE cells gradually lose their tight junctions and then begin to migrate and do not stay on Bruch`s membrane (Fig 5 d). Also, the hexagonal shape of the cells and the ability to phagocytose ROS gets lost.*
As RPE cells divide, their pigment becomes diffuse and is often released from cultured cells over time [3]. Additionally, the RPE cells of method 1 are not able to proliferate in culture, therefore, it is not possible to maintain the cells over long-term culture.

4.2.2 Method 2

The conventional cell culture (the second method) yields 30 000 ± 6300 cells per eye after 2 weeks. The cells showed a satisfying viability but the number of cells strongly fluctuated. In contrast to method 1, the cells have the ability to proliferate and the cells can reach confluence.
Furthermore, the complex preparation process, such as the need of trypsination, makes this method more time - consuming to perform than culturing entire flat mounts [3]. For the same reason this method is more expensive than method 1. Another disadvantage is, that the contamination with fibroblasts is high, which makes further experiments imprecise.

4.2.3 Method 3

The third method was ultimately the most rewarding possibility for culturing the RPE cells of adult rats [3]. The dissection process is the same as in method 1, but without further cutting the eyecup into 6 flatmounts, the whole eyecup can be cultured in a culture dish. After the cells settled on the bottom of the plastic, as in method 1, they can be subcultured and observed by Light microscopy.
The great advantage of this method was that the yield that was enhanced by the factor 10 (314 000 ± 174 000 cells per eye after 2 weeks). Moreover, the preparation process is less time-consuming than the other two methods, so the possibility to damage the cells during the preparation is reduced [3]. Because of less effort, this method is cheaper than method 2. In contrast to method 1, the cell culture is not as contaminated with fibroblasts and the cells are able to proliferate. It could be less time- consuming, if the overnight incubation would not be required. Summing up one can say that this method combines the advantages from the two other methods, if the need is to maintain the cells over long- term culture.

4.3 Comparison of all three methods

The method that attained the highest cell yield was the third method, meaning after RPE cells spontaneously detached from the flat mounts and continued to grow on the plastic. With regard to morphology, it was the first method, i.e. when RPE cells stay on their natural extracellular matrix, but only when the flat mounts were inspected directly after preparation. In contrast to methods 2 and 3, the yield of the first method reduced after further cultivation [3]. So, the cells on flatmounts possess a poor or none proliferation. However, when the cells begin to settle on a plastic bottom of the petri dish, they grow to confluence. Beside the cell yield, the third method shows furthermore advantages in cell purity. The cells from method 3 could be identified as retinal pigment epithelial cells in nearly pure culture, whereas the cells from method 2, that were isolated by the enzymatic method contained various fibroblasts, which could make future experiments inconclusive. The enzymatic removal of cells in method 2 leads to the detachment of many other cell types, beside the retinal pigment epithelial cells. Eventually, the third method is the cheapest method, because the need of eyes is very low in relation to the yield.

The differences between RPE cells originating from pigmented versus albino rats were not significant (Table 3) [3].

4.4 Tight Junctions

The function of the RPE to build the blood - retina barrier is realised by the tight junctions. These cell junctions seal the cell borders and prevent free diffusion into the neural retina [8]; [9]; [10]. To examine the morphology of the cells from all 3 methods we used an antibody against tight junction proteins ZO-1. It was successful in every method but the most distinctive shape of the tight junctions was in method 1 when the cells stay on Bruch`s membrane. The hexagonal shape was widely maintained during the first days of culture. A reason could be that the cells stay in their original and natural environment. Also, they were not changed or strained by the isolation process or by trypsinization. In general, cells of every method build tight junctions in culture, no matter if they grow on the

plastic bottom of the petri dish or on a flatmount. The different shapes are visible in figure 11 a and b.

4.5 Test substance

In addition, the influence of the test substance was examined regarding five different aspects. Cell viability, phagocytic activity the effect of repeated ROS feeding- hypertrophy, cell polarity and protein synthesis.

The viability of rat RPE cells is not influenced by treatment with 3 μM of the test substance during 14 days.

Hypertrophy was observed in individual cells after repeated feedings with ROS but was not significantly enhanced by the test substance.

The percentage of cells phagocytosing FITC-ROS was increased when the RPE cells were cultivated two weeks in the presence of 3 μM of the test substance compared to the cells cultivated without the test substance.

The ability to degrade ROS as measured by development of lipofuscin-like autofluorescence after feeding with rod outer segments also was not altered by the test substance.

Cell polarity was not influenced by the test substance treatment, because the test substance did not influence the morphology of tight junctions or apical microvilli as judged by electron microscopy and immunocytochemistry.

The protein synthesis was increased after two weeks culture in the presence of 3 μM of the test substance comparing to the control RPE cells that were not treated with the test substance. In the used assay protein synthesis, could only weakly by inhibited by cycloheximide. Thus, there are doubts concerning the specificity of this assay. By additional feeding with ROS the accumulation of inclusion bodies is enhanced but only in few numbers of cells. Endocytotic more likely than exocytic activity is enhanced after the test substance treatment.

4.6 Conclusion

The development of the improved method and culture of RPE cells from adult rats makes it possible to isolate and cultivate primary retinal pigment epithelial cells from adult rats.

The three presented methods of isolation and culture of RPE cells from adult rats do have advantages, but also disadvantages, as shown in table 3.

With regard to the cell yield, method 3 is the most successful way of culturing RPE cells [3]. This method makes it possible to cultivate RPE cells for further experiments and to multiply the number of cells.

	Method 1	***Method 2***	***Method 3***
Yield	*Low*	*moderate*	*high*
Advantages	• *Fast* • *Cheap* • *pure culture* • *preserved morphology*	• *Proliferation of RPE cells*	• *pure culture* • *high yield* • *easy and fast to perform* • *proliferation of RPE cells* • *less expensive*
Disadvantages	• *high standard deviation* • *no proliferation of RPE cells* • *not for long-term culturing*	• *elaborate procedure* • *low yield* • *contamination with fibroblasts* • *expensive*	• *miminum 2 weeks of culture is needed*

Table 3

Comparison of the three methods used for the isolation and culture of RPE cells from adult rats concerning yield, advantages and disadvantages [3].

V. Summary

Purpose: *Since adult rats are used in pre-clinical studies, and due to the necessity of investigating the side-effects of drugs on RPE cells in vitro, there is a great need for primary RPE cells from these animals. The aim of this study was to develop a reproducible and quantifiable method of isolation, culture and maintenance of adult rat RPE cells. Moreover, potential differences between RPE cells coming from albino versus pigmented rats were also investigated.*

Methods: *A total number of 180 pigmented rats and 340 albino rats aged 6-14 weeks were used. RPE cells were isolated and cultured for several weeks by using three different methods: 1) growing directly on flat mounts, 2) after enzymatic isolation, and 3) after they spontaneously detached from the flat mounts and continued to grow on the plastic. Yield, cell survival and morphological characteristics were investigated using light and electron microscopy as well as immunohistochemistry.*

Results: *Immediately after isolation (day 1), the yield of the first method was: 30 000 cells/ eye, after 2 weeks: 18 000 cells/eye and after 4 weeks: 11 000 cells/ eye. The yield of RPE cells was very low after enzymatic isolation in method 2 (day 1: 13 000 cells/eye; 2 weeks: 30 000 cells/eye; 4 weeks 38 000 cells/eye) whereas it was higher when the RPE cells spontaneously detached from the flat mounts and then continued to grow on the plastic in method three. (day 1: 30 000 cells/eye; 2 weeks: 314 000 cells/eye; 4 weeks: 659 000 cells/eye). The second method often showed contamination with fibroblasts whereas the two other methods showed pure RPE cultures. The RPE cells were able to proliferate when using the second and the third method but not when they were cultivated directly on the flat mounts (first method).*

Conclusion: *The qualitative and quantitative best method for isolating adult rat RPE cells is the culture of RPE cells which spontaneously detach from flat mounts. No differences were observed between albino and pigmented RPE cells* [3].

Zusammenfassung

Das Retinale- Pigment- Epithel (RPE) befindet sich zwischen der Aderhaut (=Choroidea) und der Netzhaut (= Retina) und besteht aus einer einzelligen Schicht hexagonal aufgebauter Zellen. Um einige Aufgaben des RPE zu nennen ist es beispielsweise ein wichtiger Aspekt, dass das RPE als Teil der Blut- Retina- Schranke, mit Hilfe seiner Tight- junctions eine Barriere Funktion zwischen subretinalem Raum und Choroidea darstellt. Außerdem werden durch das RPE abgestoßene Photorezeptorzellen phagozytiert und Lichtabsorption ermöglicht. Aufgrund dieser und weiterer Aufgaben des RPE können Störungen seiner Funktion schwerwiegende Folgen für die Retinafunktion und somit für das Sehvermögen haben.

Ziel: Vorklinische Studien haben gezeigt, dass verschiedene Medikamente häufig Nebenwirkungen auf das RPE haben. Da Ratten die meist verwendeten Versuchstiere für diese Studien sind, bedarf es einer Methode, welche es erlaubt RPE Zellen von ausgewachsenen Ratten zu kultivieren, um diese Nebenwirkungen in vitro zu untersuchen. Das Ziel dieser Arbeit war eine reproduzierbare und quantitativ bestimmbare Methode, zur Isolierung und Kultivierung von RPE Zellen ausgewachsener Ratten, zu entwickeln. Potenzielle Unterschiede von pigmentierten und albino - Ratten wurden untersucht, sowie die Auswirkungen einer Testsubstanz auf das RPE. Außerdem wurde nach einer Möglichkeit gesucht, die Adhäsion der Photorezeptoraußensegmente der Retina an den Mikrovilli des RPE zu reduzieren um die Zellen bei der Isolierung nicht zu verletzen oder abzulösen.

Methoden: Insgesamt wurden 180 pigmentierte und 340 albino - Ratten im Alter von 6-14 Wochen verwendet. Für die daraus gewonnenen Zellen wurden drei verschiedene Methoden vorgestellt um das RPE zu kultivieren:

1. Die Zellen bleiben auf der Sklera und werden als Flachpräparate (= Flatmounts) kultiviert.
2. Die Zellen werden nach einer enzymatischen Isolierungsmethode abgelöst.
3. Nachdem die Zellen sich von den Flatmounts spontan ablösen wachsen sie auf der Plastikoberfläche weiter.

Die Zellausbeute, Überlebensrate und morphologische Charakteristika wurden mit Hilfe von Licht- und Elektronenmikroskopie, sowie Immunhistochemie untersucht.

Ergebnisse: Mit der ersten Methode ließ sich eine Zellausbeute von 30 000 Zellen/ Auge an Tag 1, 18 000 Zellen/Auge nach 2 Wochen und 11 000 Zellen/ Auge nach 4 Wochen erzielen. Nach Methode zwei war die Ausbeute mit: Tag 1: 13 000 Zellen/Auge, 2 Wochen: 30 000 Zellen/Auge und nach 4 Wochen: 38 000 Zellen/Auge anfangs geringer, die Zellen waren jedoch im Gegensatz zu Methode 1 in der Lage sich zu vermehren. Das beste Ergebnis im Hinblick auf die Anzahl der Zellen lieferte Methode 3 mit Tag 1: 30 000 Zellen/ Auge; 2 Wochen: 314 000 Zellen/ Auge; 4 Wochen: 659 000 Zellen/ Auge. Die Verunreinigung der Kulturen mit Fibroblasten war in Methode 2 am höchsten und in Methode 1 am geringsten.

Schlussfolgerung: Als qualitativ und quantitativ beste Methode um RPE Zellen von erwachsenen Ratten zu isolieren, stellte sich Methode 3 heraus. Unterschiede zwischen pigmentierten und albino- Ratten wurden nicht beobachtet. Die retinale Adhäsion darf bei der Präparation der Augen auf keinen Fall vernachlässigt werden, da die Zellen andernfalls beschädigt oder zerstört werden.

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VII. Erklärung zum Eigenanteil

Sowohl die gesamte Dissertation als auch der bereits publizierte Anteil wurden im Forschungsinstitut für Augenheilkunde (Sektion für Experimentelle Vitreoretinale Chirurgie, Schleichstrasse 12/1, 72076 Tuebingen) unter Betreuung von Prof. Dr. Ulrich Schraermeyer durchgeführt.
Die konzeptionelle Planung wurde in Zusammenarbeit mit Prof. Dr. Ulrich Schraermeyer und Dr. Sylvie Julien erarbeitet.

Sämtliche Versuche wurden, nach Einarbeitung durch Monika Rittgarn, Sabine Hofmeister und Sigrid Schultheiß, von mir eigenständig durchgeführt.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir, im Literaturverzeichnis angegebenen, Quellen verwendet zu haben.

Herr Prof. Dr. Schraermeyer und Frau Dr. Julien haben Korrektur gelesen.
Bei dem angeführten Manuskript ging der Anteil von Prof. Dr. Schraermeyer sowie Dr. Julien nicht über das im Rahmen eines Betreuungsverhältnisses übliche Maß hinaus.

Die Originalveröffentlichung des bereits publizierten Anteils erschien: www.springerlink.com.
Langenfeld, A., Julien, S., and Schraermeyer, U. (2015). **An improved method for the isolation and culture of retinal pigment epithelial cells from adult rats**. Graefes. Arch. Clin. Exp. Ophthalmol. 253, 1493–1502. doi: 10.1007/s00417- 015-3011-5

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Ort, Datum   Analena Langenfeld

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