Chapter 6.

Development of an accommodating intra-ocular lens –
In vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells.
Abstract

Cataract surgery is routinely performed to replace the clouded lens by a rigid polymeric intra ocular lens unable to accommodate. By implanting a silicone gel into an intact capsular bag the accommodating properties of the natural lens may be maintained or enhanced. The implantation success of accommodating lenses is hampered by the occurrence of capsular opacification (PCO) due to lens epithelial cell (LEC) growth. In order to prevent LEC proliferation, a treatment regime using actinomycin D, cycloheximide and a. dest was developed. The effectiveness of treatment was analyzed using an in vitro, MTT-based cell culture system and an ex vivo pig eye model in which the implanted lens-in-the-bag is cultured as a whole. LEC were exposed to treatment solutions for 5 minutes, then the cells were allowed to recover and to re-colonize the substratum. MTT conversion by cells was transiently inhibited by cycloheximide dissolved in a. dest and by a. dest alone. Exposure to actinomycin D resulted in a lasting inhibition of MTT conversion and consequently cell proliferation. These in vitro data could not be fully reproduced in the ex vivo pig eye model due to essential differences between both models. Treatment with actinomycin D containing solutions, however, resulted in a nearly complete absence of cells on the capsular wall. The pig eye model is a promising approach to further evaluate the effects of peri-surgical treatment during the accommodating intra ocular lens implantation.
Introduction

Cataract surgery nowadays is a well established ophthalmologic procedure. In cataract surgery, the diseased, clouded lens (Fig. 1) is replaced by an artificial, non-accommodating lens. Typically, the implanted intra ocular lens is made of more or less rigid polymers such as polymethylmethacrylate or silicone. During the lens replacement procedure, the natural, clouded lens contents is removed through a capsulorhexis, a hole created in the anterior lens capsule. The artificial lens is positioned within the capsular bag through this opening. Although this procedure is highly successful, a major complication after removal of the lens material is the occurrence of capsular opacification on the posterior side, usually referred to as PCO (posterior capsular opacification). PCO is caused by a proliferation of remaining lens epithelial cells (LEC) that still reside on the inside of the capsular bag after lens surgery [1-3]. These cells may additionally undergo a transformation into fibroblast type cells causing shrinkage and fibrosis of the lens capsule [1;4;5].

Figure 1. Anatomy of the eye (adapted from Wayne Heim - http://members.aol.com/wayneheim/computer.htm)
Several methods to prevent PCO have been presented in literature. These include optimizing design parameters of the IOL [6;7], methods of surgery and the application of chemical agents inhibiting cell adhesion, migration and proliferation, including anti-inflammatory and cell death inducing agents. Adhesion can be interfered with by using (cyclic) RGD peptides [8-10], adhesion-blocking antibodies, disintegrins [11] or calcium antagonists such as Mibefradil [7] and EDTA [10;12]. These treatments aim at an interaction with integrin functions expressed on lens epithelial cells [13-16]. Due to their interaction with extracellular matrix molecules such as collagen, it also seems possible to change the quality or quantity of the adhesive proteins produced by the cells [17;18]. Induction of cell death, the ultimate treatment, is usually induced by agents that inhibit cellular processes necessary for cell survival. This can be achieved by the non-transient inhibition of the protein synthesis machinery by e.g. thapsigargin and ricin- and saporin-conjugates [19-23]. The use of cytostatic or cytotoxic agents such as mitomycin C [24-29], aclacinomycin A [30], hydrogen peroxide [31] and the cyclooxygenase inhibitor diclofenac [32] has also been reported. Despite all efforts, the age-related incidence of posterior capsular opacification still is unacceptably high [2]. This undoubtedly is related to a lack of a proper drug delivery in the open capsular space [33]. Fortunately, the resulting PCO can routinely be treated by destruction of the opacified capsule with a Yag-laser [2].

Apart from posterior capsule opacification after IOL implantation, implanted IOLs do not offer the possibility for accommodation. Several attempts have been described to develop accommodating IOL’s [34-37], such as by filling the capsule with a soft polymer injected through a small capsulorhexis [36]. Therewith the lens capsule (Fig. 1) remains intact and can support the implanted lens, but as a drawback, opacification can now occur both posteriorly and anteriorly, while laser treatment cannot be applied as it will affect the accommodative amplitude of the soft polymer lens. Thus aggressive chemical agents have to be used prior to injection of the polymer that effectively kill all remaining lens epithelial cells. This implies that, since cataract surgery is and should remain a relatively fast procedure, chemical action should also be completed fast, for instance within 5 min. Furthermore, treatment substances must be kept localized in the capsular bag in order to prevent damage to the surrounding ocular tissues such as the ciliary body and the cornea (see Figure 1).
In this study, actinomycin D and cycloheximide have been used. Actinomycin intercalates with DNA, resulting in a decreased RNA polymerase (DNA reading) activity inhibiting RNA synthesis. Cycloheximide reversibly inhibits protein synthesis and has frequently been used in cell biological experiments [38]. Its application can result in a cell growth arrest and eventually cell death.

The aim of this study is to assess the efficacy of two aggressive cytotoxic agents, cycloheximide and actinomycin D, in prevention of capsular opacification in the development of an accommodating IOL. To this end, an in vitro and ex vivo method will be described, based on the growth inhibition of cultured lens epithelial cells obtained from pig and rabbit.

**Materials and methods**

**A. Survival of pig and rabbit lens epithelial cells exposed to treatment solutions.**

**Cell isolation and culture** - Lens epithelial cells were isolated from pig and rabbit eye lenses. Whole pig eyes were obtained from the local slaughterhouse. They were cleaned from tissue remnants such as muscles and sterilized briefly in 70% ethanol. The lens including the capsular bag (Fig. 1) was freed from the eye while working in a sterile flow hood. The capsular bag (Fig. 1) was obtained in pieces and put in a trypsin solution. The cells were collected by centrifugation and brought into culture, which was designated LECp (Lens Epithelial Cells pig). Cells were used at passage 1 to 4. A similar procedure was set up for obtaining rabbit lens epithelial cells (LECr). New-Zealand white rabbits are routinely used in animal experiments within the Central Animal Facility of the University of Groningen, and depending on the experiments performed the eyes can be obtained at the time of sacrifice of the animals. Both cell types were kept in culture medium consisting of DMEM/F12 supplemented with 20% FCS, 1% penicillin/streptomycin, 1% L-glutamin, 1% D-glucose.

**Cell treatment** - The wells of a 96-wells plate were filled with 5000 cells per well in 100 µL growth medium. After 24 h, the medium was removed from the wells and the chemical solutions were added to the wells. These included Actinomycin D (Sigma, conc: 10, 5, 1 µg/mL) and cycloheximide (Sigma, conc: 25, 10, 5, 1 µg/mL). The
chemicals were dissolved in PBS (buffer) or a. dest. After incubation in the presence of the cytotoxic agents for 5 min, cells were incubated for periods up to 288 h at 37°C in a CO₂ incubator without further exposure to the agents.

**Cell survival assessment** - In order to determine the effects of the treatment on the survival of lens epithelial cells, an MTT-assay was carried out. The MTT-assay is based on the metabolic conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid). MTT has frequently been used to monitor the vitality or biochemical activity of cell populations and as such it has been described as a measure of cell number within a population [39]. MTT (Sigma) was dissolved in PBS at a concentration of 5mg/mL. Then 20 µL of MTT solution was added to the wells. After an incubation time of 3 hr at 37°C in the CO₂ incubator, the medium was removed, and the blue crystals were dissolved in 2-propanol. The absorbance of the obtained formazan solutions was measured at 590 nm using a 96-well plate reader. As controls, full growth medium and cells without MTT were included. All experiments were performed in triplicate.

**B. Ex vivo lens implantation in the pig eye**

**Surgical procedure – lens removal** - Pig eyes were obtained from the slaughterhouse and kept in buffer until being operated upon by an eye surgeon. For surgery, the eyes were placed on a stand under a binocular microscope with a fiberoptic, coaxial illumination. A corneal incision (Fig. 1) was made with a 3 mm stainless steel keratome and the anterior chamber (Fig. 1) filled with a highly viscous Healon GV solution (Pharmacia, Uppsala, Sweden). A second incision (paracentesis) was created with a 15-degree knife. The anterior capsule (Fig. 1) was punctured with a sharp 27G needle and a small 1.0-1.5 mm capsulorhexis was created with an Utrata forceps. Then an infusion needle, connected to an infusion bottle filled with phosphate buffered saline (PBS) and positioned at a height of 40 cm above the eye, was inserted in the anterior chamber via the paracentesis. The natural lens substance (Fig. 1) was aspirated with a 20G sterile cannula connected to a 10 cc syringe. After removal of the lens no polishing of the inside of the capsular bag (Fig. 1) was performed. The anterior chamber infusion was removed and the chamber was filled with Healon GV to protect the cornea from the following chemical treatment.
Surgical procedure – treatment of the capsular bag – Cytotoxic agents were applied dissolved in either a. dest or gel-like Healon (0.507 g of sodium-hyaluronate in 49.5 ml of a solution of the treatment substance in pure a. dest; final Na-Hyaluronate concentration 1.0% wet weight). A. dest-based solutions were injected in the collapsed capsular bag with a 2 cc syringe with a 27G cannula until the capsular bag started to expand. The solution was left in place for 5 min. Subsequently, a syringe with a 27G cannula was introduced in the capsular bag and the solution aspirated until the capsular bag collapsed.

Healon gels were also administered by injection in the capsular bag. After 5 min the anterior chamber maintainer connected to the infusion bottle with BSS was re-introduced in the eye and the Healon gel containing the toxic substance was aspirated from the capsular bag with a 20G cannula.

At this stage in both treatment variants, the Healon GV was removed from the anterior chamber and the infusion system was re-inserted in the eye. Then PBS was slowly flushed through the capsular bag by way of a 27G cannula. The infusion in the anterior chamber washed away the remainders of the cytotoxic agents and/or Healon through the corneal incision.

Surgical procedure – lens implantation – The anterior chamber infusion was removed and the chamber was filled with Healon again. A 2.7 mm-diameter custom-made silicone plug (US patent 2002/0107567 A1) was inserted into the capsular bag to prevent polymer leakage during lens refilling. A two component silicone polymer (A and B; Pharmacia, Groningen, the Netherlands) was used for lens refilling [35;36]. This material attained a Youngs modulus of 0.8 kPa after 70 min of polymerisation at 20°C. For comparison, the Young’s modulus of a 20 year-old human lens is about 1 kPa, increasing to 5 kPa after 60 years. The two components were mixed through a custom-made dual syringe system with a static mixer on top and briefly subjected to vacuum to remove air bubbles. The material was subsequently injected into the capsular bag using a 3 cc syringe with a 25G cannula. Injection proceeded until the polymer began to leak past the capsular plug. Finally the cannula was retracted and the plug was manipulated into position to close the capsulorhexis.

Lens removal and in vitro cultivation - After refilling the bag, the cornea and the iris were removed with scissors and the zonular fibres cut with Vannas scissors. Then the lens was taken from the eye and placed in culture medium at 37°C and 5% CO$_2$. 
in a humid environment for 3 weeks. Medium was regularly replaced and assessed for potential breach of sterility.

**Lens characterisation** – After 3 weeks, immunocytochemical staining was performed on whole lenses including the capsular bag. Cells were stained for nuclei (DAPI) and the intermediate filaments (anti-vimentin antibodies). Labeling was examined with confocal laser scanning microscopy, as depicted in Fig. 2. Four images were taken of the anterior and posterior sides of each lens. Images were quantified for the number of cells (nuclei) on the interior site of the capsule wall, using image processing and analysis software (LEICA confocal software, PaintShopPro 8.0, Scion Image). Implantation experiments were performed in duplicate.

**Figure 2:** Positioning of the full lens under the microscope. By applying a number of custom-made holders virtually each position on the lens can be probed with a water-immersion lens.
**Statistical analysis**

In order to determine the influence of treatment and time of incubation, analysis of variance (ANOVA) was performed. In case the F-statistic was larger than the critical value at $\alpha=0.05$, the multiple comparison procedure according to Student-Newman-Keuls was employed to determine individual differences at $\alpha=0.05$ [40].

**Results**

**A. Survival of pig and rabbit lens epithelial cells exposed to cytotoxic agents**

Lens epithelial cells derived from pig eye lenses grew well up till passage 5 with an occasional cobblestone monolayer appearance. Rabbit lens epithelial cells exhibited a fine cobblestone epithelial morphology, but stopped growing beyond passage 4. MTT conversion data (expressed as corrected absorption) for pig lens epithelial cells (LECp) are shown in Figs. 3 and 4. Cycloheximid dissolved in PBS gave a moderate to negligible inhibition relative to medium control values when applied for 5 min (Fig. 3). A 10 min treatment with this combination yielded an increased inhibition of MTT conversion relative to conversion values obtained with medium (data not shown). This inhibition was only observed during the first 24 h after exposure (data not shown). Dissolved in a. dest, however, cycloheximide was a powerful inhibitor of MTT conversion (Fig. 3). One hundred percent inhibition occurred 2 days after application. However, after 12 days cell growth returned as measured by MTT conversion. A. dest alone gave rise to full inhibition after two days in culture, followed by a return to control values after 12 days in culture (Fig. 3).
Figure 3: MTT conversion by LECp (expressed as absorption) exposed to cycloheximide dissolved in PBS, cycloheximide dissolved in a. dest and actinomycin D / cycloheximide / a. dest combinations during 5 min. The treatment groups are indicated on the X-axis. The cells were incubated for a period T of 0, 24, 48, 120 and 288 h after exposure to the treatment regimes and then supplemented with MTT for a period of 3 h. Absorption values were corrected for blank controls. The units of the numbers on the X-axis are µg/mL. The xx/yy numbers represent the actinomycin D / cycloheximide concentrations used. Statistical analysis of the data revealed that all actinomycin/cycloheximide combinations gave significantly lower MTT conversion than medium control. Furthermore, Cycloheximide in A.dest at concentrations of 25, 10 and 5 µg/mL reduced MTT conversion at time points of 0 and 48 hours. Many significant differences are present, but within the SNK table distinct groups can not be assigned due to a sliding scale. As an indicator of significant differences, the bar for cycloheximide 1 µg/mL in PBS at 288 h is significantly different from cycloheximide 10 µg/mL in PBS at 48 h (indicated by arrows).
LECp were also exposed to solutions containing actinomycin-D for 5 min. MTT conversion values after 5 days of subsequent incubation were down to zero in all variants of actinomycin D administration (Fig. 3). This coincided with the almost complete absence of cells at these time points. Prolonged incubation did not give rise to enhanced MTT activity (data not shown).

The importance of the presence of a hypotonic a. dest solution during cycloheximid application is reflected in Fig. 4, in which the results of cells exposed to serially diluted PBS solutions are shown. At a 0.1X concentration of PBS, the inhibiting effect of the hypotonic solution already disappeared.

In vitro exposure experiments were also performed with lens epithelial cells derived from rabbit eyes (LECr). Results on the MTT conversion measurements on cells after exposure to treatment are shown in Fig.5. MTT conversion was almost completely inhibited after 24 h in culture and fully inhibited after 5 days in culture as a result of 5 min treatment with actinomycin D-containing solutions.

**Figure 4:** MTT conversion by LECp (expressed as absorption) exposed to serially diluted PBS solutions with increasing hypotonicity during 5 min. The cells were incubated for a period T of 0, 24, and 120 h after exposure to the PBS regimes and then supplemented with MTT for a period of 3 h. Medium alone served as control. Absorption values were corrected for blank controls. Significant differences are indicated by *, ** and ***.
B. Ex vivo lens implantation in the pig eye

Lenses could easily be removed from the eye and were brought in a culture environment. Natural lenses started to become white within days after culturing due to precipitation of salts. The implanted lenses remained clear during the incubation period. Representative images obtained by confocal laser scanning microscopy are presented in Fig. 6. The enumeration of cells on the interior site of the capsular wall is given in Fig. 7.

In terms of treatment effects, the heterogenic cell population appearances within a capsular side overruled the similarities among duplicate experiments and gave rise to large standard deviations. The posterior capsular wall displayed less cells than the anterior wall. Often, the central posterior region had sparsely distributes cell populations. The anterior side was populated with cells also in the central region. The lenses treated with actinomycin-containing solutions contained the lowest number of
cells on both sides of the capsule. In fact, barely any cell could be detected and cell remnants were obvious (Fig. 6). In Healon-cycloheximide-a. dest samples aberrant nuclei were observed that are probably indicative of apoptotic bodies.

Figure 6: Confocal laser scanning microscopic projection images of whole lens-in-bag specimens (A-G) and bag only (H). Projections were made from image stacks up till 100 μm and include both interior and exterior cell layers. Both anterior (A, C, E) and posterior sides (B, D, F, G) were screened. Exterior cells are double-labeled (vimentin – green, nuclei – blue), interior cells are only labeled for nuclei (blue). Silicone lenses were implanted in ex-vivo pig eyes without further treatment (A,B), with 25 μm/ml cycloheximide in A.dest (C,D), or with 25 μm/ml cycloheximide in Healon (E-G). The micrograph H is taken from a capsular bag isolated from a normal pig lens. All images, except G, represent a field of view of 750 x 750 μm². The arrow in G points to apoptotic bodies.
A number of general features were observed with ex vivo lens cultures. Cells were detected both on the inner and outer side of the capsule. Cells on the outer side were stained for both vimentin (intermediate filaments) and DAPI (nuclei) (Fig. 6). Cells on the inside were only stained with DAPI, as the anti-vimentin antibodies were not able to penetrate the full thickness of the capsule. Cells on the outside displayed a heterogeneous morphology ranging from fiber-like cells to fibroblasts and seem to have originated from the equatorial rim zone with its zonula attachments, and from the plug region.

**Figure 7:** Number of cells residing on the inner capsular walls of implanted pig eye lenses after three weeks in culture. Each bar represents the average (+/- s.d.) of 8 fields of view derived from two independent experiments. Ant = Anterior side. Post = Posterior side. Databars indicated with * are significantly different from the other databars.
Discussion

In this study, effective, fast, and simple in situ approaches to prevent capsular opacification were developed in both in vitro and ex vivo studies as a preamble to a new surgical lens implantation technique. The effects of two cytotoxic agents with respect to potential prevention of capsular opacification were assessed.

Two models of assessment were used in this study: First, in an in vitro cell culture system, cells were exposed to the cytotoxic agents for 5 min, and the remaining cells were allowed to recover and re-colonize the culture wells. Second, the full surgical procedure, including a 5 min chemical treatment of the lens capsule and lens implantation, was applied to a pig eye and the lens-in-the-bag was transferred to a culture environment in order to assess re-colonization of the inner wall of the lens bag. Common features of both models are the treatment window and the presence of a natural substratum to be re-colonized. Differences are however, the geometry of the cellular layer, the presence or absence of the implanted lens, the use of Healon as a drug-delivery vehicle and the time of assessment. Considering these differences, it is not surprising that correlations between the performed in vitro and ex vivo experiments are not evident. Therefore, both methods have to be evaluated on their own merits.

In the cell culture system, the observed effects of treatment with cytotoxic agents dissolved in a. dest were reached with a 5 min treatment window only. The use of a. dest creates a hypotonic environment, which seems to be pivotal in enhancing the action of cycloheximide. In vitro, an efficient fluid exchange without a significant disturbance of the low a. dest osmolarity can be achieved. This is important because as the osmolarity of the solution moves away from zero, the efficiency of the a. dest solution diminishes rapidly (Fig. 4). The osmolarity will barely be influenced by diffusion of molecules and ions derived from damaged cells. In the ex vivo and, consequently, the clinical situation the osmolarity is expected to be significantly increased due to transport of molecules from abundantly present surrounding tissues. The technique used in this study probably is not adequate to ensure a confrontation of the lens epithelial cell layer with a true a. dest solution. The implanted lenses with fully intact capsules could be kept in culture for at least 3 weeks, and probably well beyond. This corresponds with observations that human cells can be cultured on their natural substratum, the capsular bag, in vitro in organ
culture [5;41], and that rabbit LEC can be cultured on posterior capsule explants [42]. The bag surface provides the cells with an adequate substratum for adhesion, migration and possibly proliferation, with nourishment solely by diffusion from the culture medium similar to the in vivo environment [43]. Therefore, within the ex vivo model the surviving cells can use an optimal substratum for re-colonization, as was observed on the posterior side for most of the treatment regimes. After 3 weeks in a culture environment, the a. dest-based treatment regimes had not resulted in diminished cell numbers compared to the non-treated control lens, except for actinomycin-containing solutions. A. dest-mediated lysis has been observed in situ on isolated capsulotomy specimens to prevent LEC outgrowth after two min of treatment [44]. This contradicts the results described in this paper. The explanation most likely resides in the use of perfusion/irrigation [44] versus a static filling procedure, therewith allowing osmolarity changes to occur.

The heterogeneity of the obtained images on the posterior sides reflects a migration pattern of cells from the equatorial rim towards the center of the posterior side. On the anterior side, heterogeneity in the nuclei shapes may reflect the naturally occurring presence of different morphologies along this side, ranging from quiescent to germinative to differentiating cells [43;45]. All these cells have similar telomerase activity in the healthy eye, but elevated activity in case of PCO [46]. Heterogeneity can also be caused by variations in the extent of filling [47;48]. The increased number of apoptotic bodies observed with the Healon-cycloheximide-a. dest combination indicates that cells on the capsular wall have been subject to signals inducing apoptosis. Although this can be due to cycloheximid treatment, cycloheximid alone did not result in the presence of apoptotic bodies. The Healon gel may provide a storage compartment for the drug with subsequent release towards the cell layer. Although Healon is removed after 5 min, a thin layer of this viscous gel may remain at the capsular surface.

The in vitro and ex vivo data with actinomycin D suggest that this chemical dissolved in a. dest is highly efficient in reducing cell numbers on the long term. As cells are removed from the material surface, this is probably accomplished by cell death. Remaining cells do not seem to be able to recover from the treatment. Actinomycin D seems to be an excellent candidate agent to prevent anterior and posterior capsular opacification. Due to its triple action spectrum mediating inhibition of migration, proliferation and matrix secretion, actinomycin D is an efficient overall proliferation
inhibitor especially of G2/M cells [49;50] and can induce apoptosis [49]. Actinomycin D has been used in the treatment of tumors as part of chemotherapy [49;51]. It also is one of the many drugs that has been used in trials for prevention of restenosis after stent placement or catheter-based interventions in coronary artery disease, mainly by incorporating it in drug-eluting stents [52]. The pathologies of restenosis and PCO share many characteristics, two of them being the induction of cell proliferation and cell transformation / transdifferentiation. Actinomycin has been shown to prevent smooth muscle cell proliferation, but the therapeutic window has been shown to be narrow. The powerful in vitro and ex vivo responses to actinomycin-D shown in this study suggest that the application in vivo should be very carefully performed and monitored.

Summarizing, it can be concluded that lens epithelial cell growth can be completely arrested in in vitro cell cultures by the addition of actinomycin D. Neither hypotonic shock nor cycloheximide exposure can prevent repopulation of the culture dish, although the kinetics of this repopulation are influenced by the treatment. The in vitro results can partly be translated to ex vivo models of lens implantation, during which the interior capsular bag is treated with the same agents. The culturing of complete implanted lens-in-bag entities during 3 weeks yields information about the proliferative potential of treatment-surviving lens epithelial cells in the presence of the implanted lens. The model is a suitable system for improvement of the treatment technique.

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References


