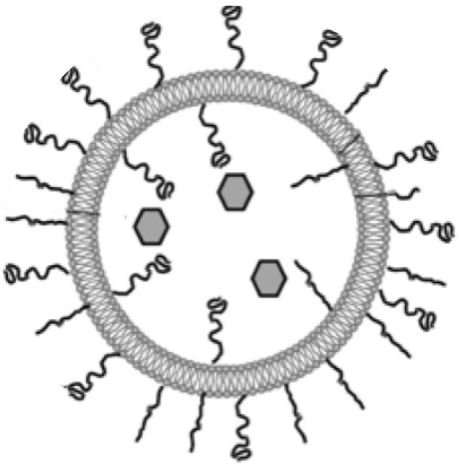

Liposomes for anti-inflammatory drug delivery in ocular diseases

From in vivo models to clinical application

Chee Wai Wong

2020

Liposomes for anti-inflammatory drug delivery in ocular diseases: From in vivo models to clinical application
Chee Wai Wong
Ph.D. Thesis, with a summary in Dutch

Surgical Retina Department, Singapore National Eye Centre
Clinical Translational Research, Singapore Eye Research Institute
Duke NUS Medical School

This research was supported by funding from the National Medical Research Council Research Training Fellowship Award, Singapore National Eye Centre's Human Research Endowment Fund, and the Singhealth Duke NUS Ophthalmology and Visual Sciences Academic Clinical Program.
May 2020

The printing of this thesis was financially supported by:
National Medical Research Council, Singapore

Liposomes for anti-inflammatory drug delivery in ocular diseases

From in vivo models to clinical application

Liposomale corticosteroïden als anti-inflammatoir geneesmiddel in oogziekten
Van in vivo modellen tot klinische toepassing

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

maandag 30 augustus 2021 des ochtends te 10.15 uur

door

Chee Wai Wong
geboren op 8 Januari 1981
te Singapore

Promotoren:

Prof. dr. G. Storm

Prof.dr. T.T. W. Wong

Copromotor:

Dr. J.M. Metselaar

Contents

1

General introduction

Introduction

Inflammation: a common disease mechanism in ocular pathology

Inflammation is one of the most common disease mechanism behind ocular pathologies. Ocular inflammation can occur after any form of ocular surgery, of which cataract surgery is the most common, with more than 30 million operations performed globally in 2019 alone.[1] Inflammation is a significant component of many ocular conditions affecting the front of the eye (anterior segment), e.g., non-infectious uveitis[2], and those affecting the back of the eye (posterior segment), e.g., proliferative vitreoretinopathy[3], diabetic macular edema[4], and cystoid macular edema secondary to retinal vein occlusion[5]. Anterior segment inflammation is an inevitable consequence of cataract surgery.[1] Such inflammation, if left unchecked, can lead to corneal edema, raised intraocular pressure, and cystoid macular edema. Therefore, inflammatory complications can have a long-lasting adverse impact on vision post-cataract surgery. These complications are no longer acceptable in an era where advances in surgical techniques and innovation have raised patients' expectations for perfect surgical outcomes from cataract surgery.[1, 6] Similarly, non-infectious uveitis, if inadequately treated, can lead to sight-threatening complications like cataract, glaucoma, and macular edema in up to 25% of patients.[7] Regarding inflammation affecting the back of the eye, proliferative vitreoretinopathy is an example of excessive inflammation following retinal detachment, leading to an aberrant healing process that results in scar tissue formation and eventual blindness.

Steroids for the treatment of anterior segment ocular inflammation

Corticosteroids are the first-choice treatment for ocular inflammation. For anterior segment inflammation, the current gold standard is topical eyedrops therapy.[1] However, several limitations exist for topical eyedrop administration:

1. Poor bioavailability (less than 5%)
2. Compliance with treatment is challenging

3. Patients often have decreased vision immediately after surgery, impairing their ability to instill eyedrops accurately[8, 9]

Over 90% of patients with ocular inflammation exhibited at least one of the following behaviors: inability to instill eyedrops accurately onto the eye, administering an incorrect amount of drops, contaminating the bottle tip with their fingers, and failing to keep hand hygiene.[10] Also, premature tapering or cessation of steroid eye drop administration can lead to a rebound in ocular inflammation, leading to eye pain, redness, and blurred vision.[11] The non-specific delivery of steroids to uninflamed ocular tissue may also result in steroid-related side effects such as raised intraocular pressure and glaucoma.[11] The ideal solution would be a drug delivery system (DDS) that can replace daily topical eyedrops by providing increased bioavailability and sustained delivery to ocular tissue while being specifically targeted towards the inflamed tissue. This is a patient-driven unmet medical need: We have previously surveyed Singaporean glaucoma patients, 80% of whom were willing to pay an equal or higher cost compared with eye drops for an alternative drug delivery system exhibiting sustained action.[12]

Steroids for the treatment of posterior segment ocular inflammation

Many retinal conditions require treatment with corticosteroids for their anti-inflammatory effect, including diabetic macular edema (DME), retinal vein occlusion related cystoid macular edema, and as an adjunct therapy for proliferative vitreoretinopathy. Though effective, there are several drawbacks. First, ocular barriers make drug penetration to the back of the eye problematic after eyedrop instillation. Figure 1 shows a schematic of the eye and the various routes of administration to bypass barriers to ocular drug penetration. Thus, invasive intravitreal injections are required to deliver sufficient drug concentrations to the retina. Intravitreal injections are associated with risks of infection, retinal detachment, glaucoma, and lens damage. Second, corticosteroids have short ocular half-lives relative to the duration of disease activity, thus necessitating repeated and frequent

intravitreal injections. This need for frequent injections increases the cumulative risks of injection-related complications, represents a tremendous treatment burden and leads to escalating costs. Third, intravitreal injection of free steroids into the posterior segment has been associated with significant adverse side effects, including cataract and raised intraocular pressure.[13]

Driven by the shortcomings of invasive and inefficient posterior segment drug delivery, there is a massive impetus for the research and development of minimally invasive, sustained action therapeutics for related diseases. Several intraocular corticosteroid-releasing implants are currently approved by the FDA to treat DME. [14, 15] However, these implants still require administration via intravitreal injection and do not address the issue of steroid-related ocular side effects, which pose significant limitations on their use. To circumvent anatomical barriers to posterior segment drug delivery, several drug delivery technologies have been developed. Among these, nanoparticles such as liposomes, dendrimers, polymeric micelles, conjugation of therapeutic payload with cell-penetrating peptides, and port delivery systems have shown the most promise.

Liposomes as a drug delivery system for corticosteroids

Liposomes are vesicles consisting of lipid bilayers, ranging in size from 0.01 to 10 μm, composed of primarily phospholipids and cholesterol surrounding one or more aqueous internal compartments. Features of liposomes that confer advantages for their a drug delivery potential for the eye include: 1. Their amphipathic nature allows the incorporation of both hydrophobic and hydrophilic drugs. A single liposome particle may carry more than one drug drugs, allowing the simultaneous delivery of combination drug therapy, exemplified by the commercial liposome product Vyxeos.[16, 17] 2. Liposomes are biocompatible and biodegradable.[18] 3. Surface conjugation of ligands to liposomes can facilitate targeted binding to desired receptors.[19] 4. Surface PEGylation (conjugation of polyethylene glycol to the liposome surface) is a popular strategy to improve dispersibility, increase stability, and

reduce clearance.[20] 5. Liposome encapsulation can protect the encapsulated drug from degradation and increase the intravitreal half-life of the drug by 7-11 times depending on the encapsulated drug.[21, 22] 6. The surface charge and particle size of liposomes can be altered to enhance the penetration through the sclera.[23]

Liposomes have proven to be a successful drug delivery system for treating ocular diseases and have made it to human clinical trials, involving their application to treat dry eyes[36], allergic rhinoconjunctivitis[37] and cytomegalovirus infection of the retina[38]. In earlier studies, Pouvreau et al[22] and Broekhuyse et al[21] have observed a significant anti-inflammatory effect in experimental uveitis after depletion of macrophages with dichloromethylene diphosphonate (Cl2MDP)-containing liposomes. These results suggest that liposomes may have the advantage of preferential uptake by macrophages, the primary cell type involved in anterior uveitis, as demonstrated in our study with the observation of co-localization of liposomes and macrophages (see Chapter 4).

While the application of steroids is crucial after ocular surgery for the adequate suppression of inflammation-related sight-threatening complications such as raised intraocular pressure and cataracts, steroids themselves may also cause these side effects. Previous studies have shown that 30% of all steroid-treated eyes may experience an elevation of intraocular pressure after prolonged topical steroid treatment. This side effect is related to a direct impact of steroids on the extracellular matrix proteins in the trabecular meshwork and the inhibition of phagocytosis by trabecular meshwork cells, both of which cause a reduction in aqueous outflow.[39,40] Preferential uptake of liposomes by macrophages may reduce the total dose required for sufficient efficacy by concentrating the release of the encapsulated drug in inflamed tissue only. Besides, the encapsulation of steroids within liposomes may mitigate some of their harmful side effects on the trabecular meshwork. With respect to induction of cataract formation, encapsulation of the steroid may avoid this adverse effect, Accumulation of liposomes within inflammatory sites may also have the benefit of an overall lowering of the steroid dose.

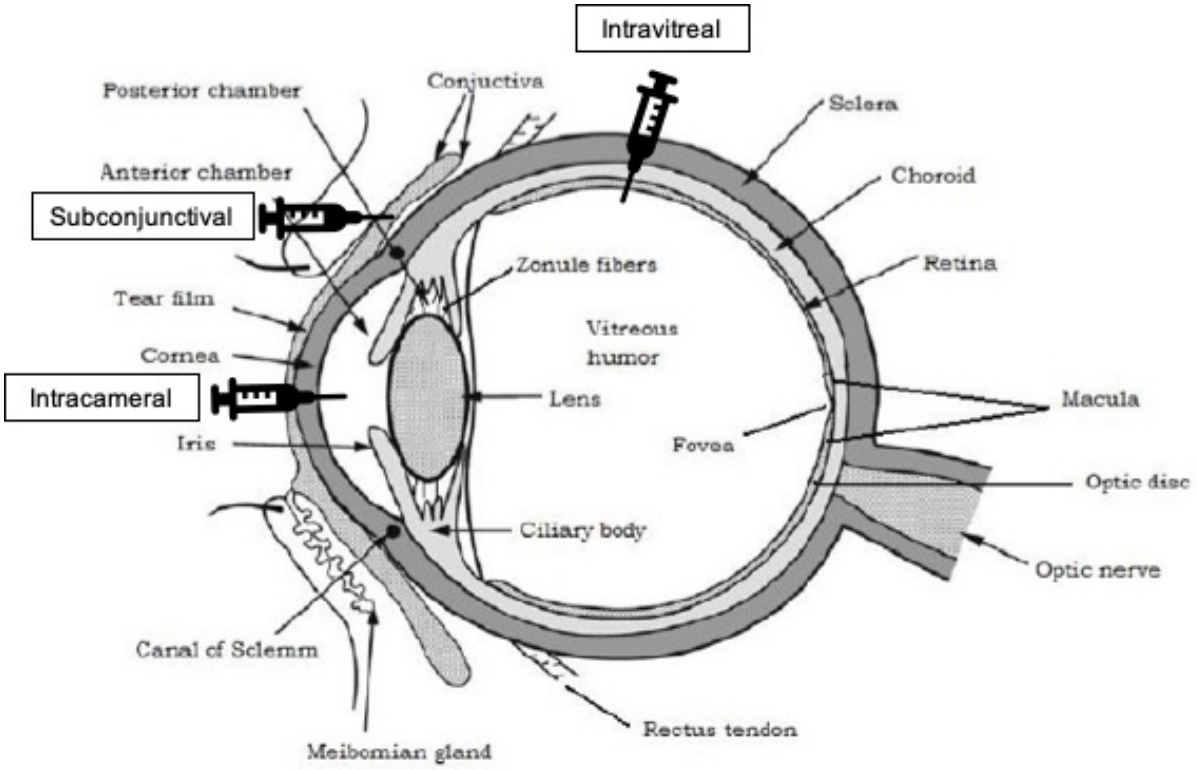

Figure 1: A schematic of the eye showing the different routes of drug administration, other than topical eyedrop application, to bypass some of the ocular barriers to drug penetration. Injections can be performed into the vitreous (intravitreal), into the anterior chamber (intracameral) or into the subconjunctival space.

Objectives and outline of the thesis

The aim of this thesis is to explore the therapeutic utility of corticosteroid-containing liposomes administered subconjunctivally for the treatment of ocular inflammation in the anterior segment as well as posterior segment of the eye..

In **Chapter 2**, we provide an overview of the current clinical applications of drug delivery systems for the treatment of ocular inflammation in the anterior segment. The various drug delivery systems containing anti-inflammatory drugs developed for this purpose are listed and summarized in this review,

with a focus on commercially available products and those in clinical trials or late-stage preclinical development.

In **Chapter 3**, we provide a review on posterior segment drug delivery systems that are commercially available or in late-stage clinical development, with a focus on treatments for exudative age-related macular degeneration and diabetic macular edema, the most common posterior segment diseases that require long term treatment. We discuss the relative merits and downsides of each drug delivery system for overcoming ocular barriers and achieving less invasive administration routes than the current standard of care i.e. intravitreal injection.

Chapter 4 reports on the in vivo efficacy of liposomal steroids, given as a single subconjunctival dose, in a rabbit model of anterior segment uveitis. We have compared the effectiveness of liposomal steroids with topical eye drops, and with subconjunctival injection of free steroids, in controlling experimental uveitis over 30 days.

Proliferative vitreoretinopathy (PVR) is a complex disease with pathogenic mechanisms that are not well understood. Developing a clinically relevant animal model to evaluate the use of liposomal steroids for this condition is essential. In **Chapter 5**, a rabbit model of PVR is assessed for reproducibility and similarity with the human disease. We have evaluated the cytokine and growth factor profiles in the vitreous humor in this PVR model to better understand the temporal sequence of pathogenic events in PVR. In addition, the limitations of this animal model are discussed.

Optimization of the preclinical in vivo model can be required for the evaluation of experimental therapeutics. In **Chapter 6**, we address the limitations of the in vivo model from Chapter 5 by comparing two methods of inducing PVR in the minipig. Minipig eyes have an anatomy that is closer to that of the human eye, compared to rabbits. This chapter describes a modification of a pre-existing model of PVR in the pig eye, to achieve closer modeling of the disease process seen in the human eye.

In **Chapter 7**, we have assessed the efficacy of a single intravitreal injection of liposomal steroids for reducing the severity of PVR in the animal model described in Chapter 6. Based on the findings in Chapter 5, we have determined the optimal timing of anti-inflammatory treatment. We briefly discuss previous clinical studies of steroid use in PVR and the advantages that a liposomal drug delivery system might have over free steroids in adjunctive PVR therapy.

In **Chapter 8**, we present the results of a first in man Phase I/II clinical trial for the use of liposomal prednisolone phosphate as an anti-inflammatory treatment for post-cataract surgery inflammation, given as a single subconjunctival dose during cataract surgery.

Finally, in **Chapter 9**, we summarize and discuss the findings in the thesis and offer perspectives for further clinical development.

References

1. Liu YC, Wilkins M, Kim T, Malyugin B, Mehta JS. Cataracts. *Lancet* 2017;390:600-612.

2. Hsu YR, Huang JC, Tao Y, et al. Noninfectious uveitis in the Asia-Pacific region. *Eye (Lond)* 2019;33:66-77.

3. Pastor JC, Rojas J, Pastor-Idoate S, Di Lauro S, Gonzalez-Buendia L, Delgado-Tirado S. Proliferative vitreoretinopathy: A new concept of disease pathogenesis and practical consequences. *Prog Retin Eye Res* 2016;51:125-155.

4. Tang J, Kern TS. Inflammation in diabetic retinopathy. *Prog Retin Eye Res* 2011;30:343-358.

5. Daruich A, Matet A, Moulin A, et al. Mechanisms of macular edema: Beyond the surface. *Prog Retin Eye Res* 2018;63:20-68.

6. McColgin AZ, Heier JS. Control of intraocular inflammation associated with cataract surgery. *Curr Opin Ophthalmol* 2000;11:3-6.

7. de Smet MD, Taylor SR, Bodaghi B, et al. Understanding uveitis: the impact of research on visual outcomes. *Prog Retin Eye Res* 2011;30:452-470.

8. Schwartz GF, Hollander DA, Williams JM. Evaluation of eye drop administration technique in patients with glaucoma or ocular hypertension. *Curr Med Res Opin* 2013;29:1515-1522.

9. Tatham AJ, Sarodia U, Gatrad F, Awan A. Eye drop instillation technique in patients with glaucoma. *Eye (Lond)* 2013;27:1293-1298.

10. An JA, Kasner O, Samek DA, Levesque V. Evaluation of eyedrop administration by inexperienced patients after cataract surgery. *J Cataract Refract Surg* 2014;40:1857-1861.

11. Renfro L, Snow JS. Ocular effects of topical and systemic steroids. *Dermatol Clin* 1992;10:505-512.

12. Chan HH, Wong TT, Lamoureux E, Perera S. A Survey on the Preference of Sustained Glaucoma Drug Delivery Systems by Singaporean Chinese Patients: A Comparison Between Subconjunctival, Intracameral, and Punctal Plug Routes. *J Glaucoma* 2015;24:485-492.

13. Wong CW, Wong TT. Posterior segment drug delivery for the treatment of exudative age-related macular degeneration and diabetic macular oedema. *Br J Ophthalmol* 2019;103:1356-1360.

14. Grewal DS, Fletcher DC, Hariprasad SM, Suner IJ. Effect of fluocinolone acetonide 0.2 mug/day implant on the decision to drive in patients with diabetic macular oedema: a report from the FAME study. *BMJ Open Ophthalmol* 2019;4:e000405.

15. Boyer DS, Yoon YH, Belfort R, Jr., et al. Three-year, randomized, sham-controlled trial of dexamethasone intravitreal implant in patients with diabetic macular edema. *Ophthalmology* 2014;121:1904-1914.

16. Madni A, Sarfraz M, Rehman M, et al. Liposomal drug delivery: a versatile platform for challenging clinical applications. *J Pharm Pharm Sci* 2014;17:401-426.

17. Lancet JE, Uy GL, Cortes JE, et al. CPX-351 (cytarabine and daunorubicin) Liposome for Injection Versus Conventional Cytarabine Plus Daunorubicin in Older Patients With Newly Diagnosed Secondary Acute Myeloid Leukemia. *J Clin Oncol* 2018;36:2684-2692.

18. van Rooijen N, van Nieuwmegen R. Liposomes in immunology: multilamellar phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without any immunogenic activity of its own. *Immunol Commun* 1980;9:243-256.

19. Ahmad I, Allen TM. Antibody-mediated specific binding and cytotoxicity of liposome-entrapped doxorubicin to lung cancer cells in vitro. *Cancer Res* 1992;52:4817-4820.

20. Milla P, Dosio F, Cattel L. PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. *Curr Drug Metab* 2012;13:105-119.

21. Barza M, Stuart M, Szoka F, Jr. Effect of size and lipid composition on the pharmacokinetics of intravitreal liposomes. *Invest Ophthalmol Vis Sci* 1987;28:893-900.

22. Gupta SK, Velpandian T, Dhingra N, Jaiswal J. Intravitreal pharmacokinetics of plain and liposome-entrapped fluconazole in rabbit eyes. *J Ocul Pharmacol Ther* 2000;16:511-518.

23. Joseph RR, Tan DWN, Ramon MRM, et al. Characterization of liposomal carriers for the trans-scleral transport of Ranibizumab. *Sci Rep* 2017;7:16803.

2

A review of the clinical applications of drug delivery systems for the treatment of ocular anterior segment inflammation

Chee Wai Wong[1,2,3]
Josbert M. Metselaar[5]
Gert Storm[4,6*]
Tina T. Wong[1,2,3*]

1. Singapore National Eye Centre (SNEC), 11 Third Hospital Avenue, Singapore 168751
2. Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore 168751
3. Duke-NUS Graduate Medical School, 8 College Rd, Singapore 169857
4. Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands.
5. Department of Experimental Molecular Imaging, University Clinic and Helmholtz Institute for Biomedical Engineering, RWTH Aachen University, Aachen, 52074, Germany.
6. Department of Biomaterials Science and Technology, Technical Medical Centre, Faculty of Science and Technology, University of Twente, Enschede, the Netherlands

Br J Ophthalmol. 2020 Oct 30:bjophthalmol-2020-315911. Epub ahead of print.

Abstract

Ocular anterior segment inflammation is a medical problem that is seen in cases of cataract surgery and non-infectious anterior uveitis. Inadequately treated anterior segment inflammation can lead to sight threatening conditions such as corneal edema, glaucoma and cystoid macular edema. The mainstay of treatment for anterior segment inflammation is topical steroid eyedrops. However, several drawbacks limit the critical value of this treatment, including low bioavailability, poor patient compliance, relatively difficult administration manner, and risk of blurring of vision and ocular irritation. A drug delivery system (DDS) that can provide increased bioavailability and sustained delivery while being specifically targeted towards inflamed ocular tissue can potentially replace the daily eyedrops as the gold standard for management of anterior segment inflammation. The various DDS for anti-inflammatory drugs for the treatment of anterior segment inflammation are listed and summarized in this review, with a focus on commercially available products and those in clinical trials. Dextenza, INVELTYS, Dexycu and Bromsite are examples of DDS that have enjoyed success in clinical trials leading to FDA approval. Nanoparticles and ocular iontophoresis form the next wave of DDS that have the potential to replace topical steroids eyedrops as the treatment of choice for anterior segment inflammation. With the current relentless pace of ophthalmic drug delivery research, the pursuit of a new standard of treatment that eliminates the problems of low bioavailability and patient compliance may soon be realised.

Introduction

The most common forms of ocular inflammation in the front of the eye are post cataract surgery inflammation and non-infectious uveitis. Anterior segment inflammation is an inevitable consequence of cataract surgery which, if left unchecked, can lead to corneal edema, raised intraocular pressure and cystoid macular edema. These complications are no longer acceptable in an era where advances in surgical techniques and innovation have raised patients' expectations for perfect surgical outcomes from cataract surgery.[1 2] Similarly, non-infectious uveitis, if inadequately treated, can lead to sight threatening complications like cataract, glaucoma and macular edema in up to 25% of patients.[3] Corticosteroid eyedrops are the first-choice treatment for post-cataract surgery anterior segment inflammation,[2] but several limitations exist for topical eyedrop administration. including poor bioavailability (less than 5%), compliance with treatment is challenging, and patients often have decreased vision immediately after surgery, impairing their ability to instil eyedrops accurately[4 5]

A recent study of postoperative cataract patients demonstrated that over 90% of patients exhibited at least one of the following behaviours: inability to instil eyedrop accurately into the eye, instilling an incorrect amount of drops, contaminating the bottle tip, and failing to realise hand hygiene before instillation.[6] In addition, premature tapering or cessation of steroid eyedrops can lead to a rebound in ocular inflammation, leading to eye pain, redness and blurred vision.[7] The untargeted delivery of steroids to uninflamed ocular tissue may also result in steroid related side effects such as raised intraocular pressure and glaucoma.[7] The ideal solution would be a drug delivery system (DDS) that can replace daily topical eyedrops by providing increased bioavailability and sustained delivery to ocular tissue while being specifically targeted towards the inflamed tissue.

It is clear from recent reviews on ocular drug delivery in the published literature that many early DDS never progressed beyond early stage preclinical development, illustrating the daunting challenges these systems face en route to clinical development. In this review, we present the applications of

drug delivery systems that are commercially available and those currently in late stage clinical trials for the treatment of anterior segment ocular inflammation with a focus on steroids and NSAIDs, drugs that are currently used as topical eyedrops for the treatment of ocular inflammation.

Nanoparticles

Liposomes

Liposomes are vesicles consisting of lipid bilayers, ranging in size from 0.01 to 10 μm, composed primarily of phospholipids and cholesterol surrounding an aqueous internal compartment. Liposomes have several characteristics of an ideal ocular drug delivery system. First, they are completely biocompatible and biodegradeable; Second, the lipophilic exterior of liposomes allows greater penetration through ocular barriers; Third, their amphipathic nature provides flexibility for the incorporation of both hydrophobic and hydrophilic drug molecules; Fourth, liposomal encapsulation decreases drug elimination, thereby increasing ocular bioavailability; Fifth, surface modification of liposomes can further enhance solubility and facilitate their passage through the various ocular compartments.[8-14] Our group studied the effectiveness of liposomal steroids, administered as a single injection in the subconjunctival space, for the treatment of experimental uveitis in a rabbit model of anterior uveitis.[15] After induction of uveitis (Day 0), rabbits were allocated to one of 5 treatment groups (pred forte eyedrops 4 times a day, subconjunctival free triamcinolone acetonide, subconjunctival liposomal prednisolone phosphate, subconjunctival liposomal triamcinolone acetonide, controls) and received treatment based on allocation on Day 3. Rabbits that received a single injection of subconjunctival liposomal steroids had significantly lower inflammatory scores (3-6 fold greater reduction in inflammatory score) than untreated rabbits on Day 4 and Day 8 after induction of uveitis, and 2-fold greater reduction in inflammatory score than rabbits given steroid eyedrops 4 times a day for 14 days on Day 8 ($p=0.03$). The subconjunctival liposomal steroid groups continued to have greater suppression of inflammation than untreated rabbits after a repeat induction of uveitis on Day 8. Subconjunctival injection of free steroid achieved an anti-inflammatory effect that was similar to liposomal steroids on Day 8 ($p=0.02$ compared with controls). However, this anti-inflammatory effect of free steroid could not be sustained beyond Day 8, with subconjunctival liposomal steroid achieving 1.3-1.5 fold greater reduction in inflammatory score than subconjunctival

free steroid on Day 11, 3 days after the repeat induction, although this difference was not statistically significant. Immunohistochemical analyses showed that liposomes accumulated in the iris and ciliary body (the primary inflamed sites in anterior uveitis), were also found within macrophages and persisted in the eye for at least 1 month. In a Phase 1/2 non-comparative trial for the treatment of post cataract surgery ocular inflammation, a single subconjunctival injection of 0.1ml of liposomal prednisolone phosphate was performed at the end of cataract surgery in 5 eyes of 5 patients. The proportion of patients with AC cell grading of 0 was 0%, 80%, 80% and 100% at day 1, week 1, month 1 and month 2 after cataract surgery, respectively. None of the subjects required additional steroid treatment and no ocular or non-ocular adverse events were observed.[16 17]

Polymeric Nanoparticles

Polymeric nanoparticles are a diverse class of colloidal polymers with diameters ranging from 1-1000nm. Polymeric nanoparticles have great flexibility and advantages as drug delivery systems, including 1. The ability to adhere to the precorneal ocular surface by forming gels, thereby increasing drug residence time[18], 2. Sustained and controlled drug release 3. The ability to carry both hydrophilic and hydrophobic drugs, 4. Biodegradability, 5. Modifiable chemical and physical properties for the optimization of drug delivery to ocular target sites.[19]

KPI-121 1.0% (INVELTYS™)

INVELTYS™ (KPI-121 1.0%, Kala Pharmaceuticals) is a nanosuspension of loteprednol etabonate (LE) delivered by a proprietary nanoparticle-based formulation referred to as Mucus Penetrating Particles (MPP). MPPs are typically generated by rendering the surfaces of particles non-mucoadhesive e.g. by coating with low molecular weight polyethylene glycol.[20] In ex vivo preclinical studies, MPP was found to have the following attributes that favours drug delivery to the eye: even distribution of drug particles on mucosal surfaces (cervicovaginal mucus), enhanced diffusion coefficient of drug particles through mucus compared to uncoated particles and prevents drug particles from becoming entrapped and transported away from its intended target by the mucus

layer.[21] In KPI-121, LE is coated with a non-covalently adsorbed surface-altering agent, Pluronic.RTM.F127, which comprises of a triblock copolymer, poly(ethylene oxide))-(poly(propylene oxide))-(poly(ethylene oxide)). A preclinical pharmacokinetics study of LE-MPP showed a 3 fold higher C_{max} in the cornea, iris/ciliary body, aqueous humor and retina compared to LE, demonstrating the increased ocular bioavailability conferred by MPPs.[22] These properties allow KPI-121 to efficiently penetrate the mucin layer of the ocular tear film and enhance drug release to the underlying tissue.

In 2 multicentred, randomized controlled clinical trials (NCT02163824 and NCT02793817), 386 subjects were treated with KPI-121 1% and 325 were treated with placebo following cataract surgery. Each group was dosed twice a day for 2 weeks. Primary outcome measures were complete resolution of ocular inflammation and complete resolution of subject-rated ocular pain at Days 8 and 15 with no rescue medication before Day 15. KPI-121 1% was shown to be significantly better than placebo for both primary outcome measures. Adverse events were reported more frequently with placebo than KPI-121.[23]

RX-10045

Micelles (10– 100 nm) consist of self-assembling, amphiphilic molecules or block copolymers and have a hydrophobic core and a hydrophilic shell. They can solubilize hydrophobic drugs by encapsulation within the hydrophobic core and it has been claimed that their small size and surface hydrophilicity allow efficient penetration of ocular barriers.[24] RX-10045 nanomicellar solution (Auven Therapeutics) is an aqueous micellar dispersion of an isopropyl ester prodrug of resolvin E1. Resolvins are a group of molecules derived from omega-3 fatty acid that can exert anti-inflammatory effects in very low dose ranges *in vitro* and *in viv*o. Preclinical testing of topical RX-10045 in new Zealand albino rabbits demonstrated appreciable drug concentration in the anterior segment ocular tissue and its active metabolite, RX-10008, was also observed in the retina/choroid. The formulation was well tolerated with no detectable ocular tissue damage.[25] A Phase 2 randomized clinical trial was performed (NCT02329743) to assess the safety and efficacy of 2 concentrations of RX-10045

(0.05% and 0.1%) compared to placebo for the treatment of post cataract surgery pain and ocular inflammation. The trial recruited 256 participants with a 1:1:1 randomization. Both formulations of RX-10045 were not significantly better than the placebo group in achieving the primary endpoint of clearing anterior inflammation at day 8 post cataract surgery (22.8% in both treatment groups compared to 16.7% in the placebo group). Similarly, RX-10045 was not better than placebo in controlling post-operative pain: the proportion of patients with no ocular pain on day 3 post-surgery was 31.6%, 26.6% and 42.3% in the 0.05%, 0.1% and control groups respectively.[26] It was postulated that the presence of efflux transporters, expressed on the ocular surface, such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and breast cancer-resistant protein (BCRP) reduced the ocular penetration of RX-10045. In addition, RX-10045 was found to be a strong inhibitor of organic cationic transporter-1 (OCT-1), further limiting its ocular bioavailability.[27]

On the other hand, OTX-101 0.09%, a novel, nanomicellar, clear aqueous solution of cyclosporine developed for the treatment of dry eye disease (Cequa; Sun Pharmaceutical Industries, Cranbury, NJ) has obtained FDA approval after a successful Phase 3 study that enrolled 744 patients. A significantly larger proportion of eyes in the treatment group achieved the primary endpoint of an increase of 10mm or more in the Schirmer test score, an objective measurement of the severity of dry eye, compared to the control group at day 84 (16.6% vs 9.2%, $p<0.001$).[28] Of particular interest was that a preclinical pharmacokinetics study showed that a single topical instillation of OTX-101 0.05% resulted in extensive distribution of cyclosporine into ocular tissues. The greatest drug concentration was in the conjunctiva, tears and sclera, but significantly higher concentrations were also found in the iris/ciliary body and aqueous humor with OTX-101 compared to application of free cyclosporine, suggesting enhanced intraocular penetration with the nanomicellar formulation.[29]

Bromfenac Durasite

DuraSite® technology (Sun Pharma, Alameda, CA, USA) represents a mucoadhesive ocular DDS consisting of a synthetic polymer of cross-linked polyacrylic acid and polycarbophil, and contains

small drug molecules in an aqueous matrix. DuraSite increases ocular residence time of the drug formulation, delivered in the form of an eyedrop, by increasing viscosity and bioadhesion to the ocular surface. Both clinical and nonclinical studies have shown DuraSite drug delivery system to be safe and nontoxic. The Durasite technology was utilised to deliver Bromfenac, a potent topical nonsteroidal anti-inflammatory drug (BromSite), for the treatment of postoperative inflammation and ocular pain. A multicentre, randomized, double-masked, vehicle-controlled, parallel-group clinical trial (NCT01576952) was conducted to evaluate the ocular safety, tolerability, and efficacy of topical administration of bromfenac 0.075% in Durasite (BromSite) compared to vehicle when dosed twice a day beginning 1 day prior to cataract surgery, the day of surgery, and then continuing for 14 days after surgery.

At Day 15, a greater proportion of subjects in the BromSite group as compared to the vehicle group had complete resolution of inflammation in terms of anterior chamber cells (57.1% vs 18.8%, respectively; $P<0.00$). More BromSite-treated subjects had no pain compared with the vehicle-treated subjects ($P<0.001$). The trial demonstrated the safety and efficacy of BromSite in reducing inflammation and pain after cataract surgery, leading to FDA approval in April 2016.[30]

DexaSite

DexaSite is a formulation of dexamethasone in DuraSite 2, which uses the same polycarbophil polymer in DuraSite with the addition of a second polymer, Chitosan to achieve greater viscosity when applied on the eye compared with DuraSite. DuraSite 2 was shown in a preclinical study to achieve the highest mean concentration of ketorolac tromethamine in the aqueous humor when applied topically to rabbit eyes, compared to DuraSite or free ketorolac.[31] In a randomized controlled Phase 3 clinical trial (NCT03192137) (n=260), a significantly larger proportion of study participants who received treatment with DexaSite applied twice daily for 16 days after cataract surgery had absence of anterior chamber inflammation at Day 15 compared to those who received vehicle (47.9% vs 22.2%, $p<0.001$).[32]

OCS-01

OCS-01 (Dexamethasone Cylcodextrin Nanoparticle Ophthalmic Suspension 1.5% mg/ml) is an eyedrop formulation of dexamethasone with cyclodextrin designed to treat inflammation and pain following cataract surgery. Cyclodextrins are water-soluble oligosaccharides which form complexes with the lipophilic, water insoluble dexamethasone, thereby increasing the solubility of dexamethasone without affecting it's lipophilicity. The complexation allows dexamethasone to retain its ability to permeate lipophilic ocular barriers i.e. the cornea. The hydrophilic cyclodextrin molecules are unable to penetrate the cornea and are subsequently washed out of the ocular surface by tears. In the recently announced topline results from a randomized, vehicle controlled Phase 2 trial (NCT04130802) in 153 patients following cataract surgery, OCS-01 applied once a day achieved a higher percentage of eyes with absence of anterior chamber inflammation (51% vs 19.6%, $p<0.001$) and a higher percentage of eyes with no pain (72.5% vs 54.9%, $p=0.005$) compared to vehicle at day 15.[33 34]

Intraocular implants

Surodex

Surodex (Oculex Pharmaceuticals Inc, Sunnyvale, California) is an intraocular implant composed of a biodegradable lactic acid/glycolic acid copolymer and loaded with 60ug of dexamethasone. This biodegradeable matrix, measuring 1.0x0.5mm in size, provides sustained release of dexamethasone for up to 10 days after implantation into the anterior chamber of the eye. In the case of eyedrops, however, drug levels decline to non-therapeutic levels within hours of 0.1% dexamethasone eyedrop instillation.[35]

A randomized clinical trial was performed on 60 eyes undergoing extracapsular cataract extraction with intraocular lens implantation.[36] In this trial, Surodex was inserted in the anterior chamber via surgical incision at the conclusion of surgery in the intervention group while the control group

received dexamethasone 0.1% eyedrops 4 times a day for 4 weeks. The study found significant reductions in anterior chamber flare in the surodex group from day 4 post operatively on. At 3 months post-surgery, flare reduction to preoperative levels were still seen in the Surodex group while the control eyedrop group still had raised flare levels. Therapeutic failure, defined as a need for augmentation of steroids, was seen in 5 (17.9%) eyes in the control group and 1 (3.1%) in the Surodex group. Lastly, the safety profile of Surodex was acceptable with no eyes developing glaucoma and no significant endothelial cell loss at 1 year post surgery. Oculex Pharmaceuticals was later acquired by Allergan in 2003. Phase III trials were never conducted and Surodex never made it to the commercial market. This was largely attributed to the challenge of obtaining Medicare reimbursement for Surodex at that time. Since then, medicare reimbursement for newly FDA-approved medical devices and drugs has been facilitated by the conferment of transitional pass-through status, which boosts patient access to these innovative therapies.

Dexycu

The Dexycu® (Icon Bioscience, Inc. Sunnyvale, CA, USA) treatment is applied as a single intracameral injection at the end of cataract surgery using Icon's Verisome™ (Icon Bioscience, Inc.) drug delivery technology to dispense a biodegradable extended-release DDS formulation of dexamethasone. The Verisome™ proprietary DDS technology allows the formulation of drugs into a slightly viscous gel, which when injected into the eye, coalesces to form a spherule that releases the drug over time. In early 2018, Dexycu was the first long-acting intracameral product to be approved by the FDA in the US for treating inflammation following cataract surgery.

A randomized, double masked multicentre trial (NCT02547623) recruited 394 patients and randomized 1:2:2 into 3 arms: 1. 5µl injections of placebo 2. 5µl injections of 342ug Dexycu and 3. 5ul injections of 517µg Dexcyu into the anterior chamber at the end of cataract surgery. Patients were followed for 90 days after surgery. At post-operative day 8, resolution of anterior chamber cells was achieved in 25.0%, 63.1% and 66.0% of eyes in the placebo, 342ug and 517µg treatment groups, respectively ($P < 0.001$). Complete resolution of clinical signs of anterior segment inflammation

(Anterior chamber cell + flare) at post-operative day 8 was achieved in 33.8%, 63.1% and 67.3% of eyes receiving placebo, 342ug and 517µg Dexycu, respectively (P < 0.001). The safety profile of both Dexycu doses were similar to placebo and no serious ocular adverse events were reported for the whole 90-days observation period.[37 38]

Dextenza

Dextenza (Ocular Therapeutix, Inc, Bedford, Massachusetts) is the first FDA-approved intracanalicular insert to deliver dexamethasone for the treatment of postoperative ocular pain with one treatment for up to 30 days. The insert contains 0.4 mg of dexamethasone.[39] The implant's proximity to the ocular surface allows maintenance of sufficient drug concentration, increasing bioavailability from less than 5% (eyedrops) to more than 70%. The safety and efficacy of Dextenza were assessed in a multicentred randomized double-masked placebo-controlled phase 3 trial (NCT02089113) in which 218 adult patients undergoing cataract surgery received Dextenza implant and 222 received placebo. At Day 14, significantly more patients had resolution of anterior chamber cells in the Dextenza arm compared with placebo (52.3% versus 31.1%; *P* < .0001). Rescue therapy was required in twice as many in the placebo arm than in the treatment arm at Day 14. Dextenza was well-tolerated, with a safety profile similar to that of placebo.[40]

Nepafenac Punctal Plug Delivery System[23]

The Nepafenac Punctal Plug Delivery System (N-PPDS) is a L-shaped, non biodegradeable, medical grade silicone punctal plug with a drug eluting core that contains nepafenac, a non-steroidal anti-inflammatory drug. The punctal plug is designed to be inserted into the lower punctum, releasing nepafenac consistently over a month, after which the plug is removed from the punctum. A Phase 2, multi-centre, randomized, parallel-arm, double-masked, placebo-controlled study (NCT03496467) was conducted to study the safety and efficacy of N-PPDS. 50 patients had an N-PPDS inserted in the lower punctum of their scheduled surgical eye, 1-2 days prior to surgery, while 25 study patients had a placebo punctal plug inserted. These plugs were retained for a period of 2 weeks following cataract surgery. The study has completed recruitment but results are yet to be released.[41]

DSP-Visulex

DSP-Visulex is a reloadable dexamethasone sodium phosphate (DSP) drug delivery system that combines a highly concentrated DSP solution with an ocular drug applicator (Visulex). The applicator consists of a medical grade silicone polymer shell shaped like a contact lens and an annular white sponge, fabricated with a proprietary sponge material, along the rim of the applicator. DSP is injected into a drug loader where the applicator is docked, facilitating the permeation of the drug into the sponge. The applicator is then placed carefully over the centre of the eye such that only the sclera is in contact with the sponge.[42] After a single application, the concentration of DSP in most of the ocular tissues, including cornea, sclera, conjunctiva, retina-choroid, and anterior chamber, was significantly higher than 1 mg/g which was deemed to be the minimum effective concentration of DSP. The highest concentration of DSP in ocular tissues was within the cornea, followed by the sclera, conjunctiva, retina-choroid, anterior chamber, lens and was lowest in the vitreous. DSP concentration, except in the lens and vitreous, correlated well with both increasing the concentration of DSP loaded in the Visulex system, and the duration of treatment.[43] A randomized Phase I/II clinical trial was performed to assess the safety and efficacy of a 5 minute application of DSP Visulex (8% and 15% intervention arms, given twice in the first week and then weekly thereafter) compared to daily prednisolone acetate 1% for non-infectious anterior uveitis (NCT02309385). At Day 29 of treatment, 90%, 88% and 77% of patients had resolution of anterior chamber cells in the 8% DSP-Visulex, the 15% and prednisolone acetate eyedrop groups respectively. More adverse events were seen in the 15% group, which included headache, eye pain, corneal abrasion, conjunctivitis and keratitis, all of which resolved. IOP elevation was not observed after day 3 in the DSP-Visulex groups.[44]

Ocular iontophoresis

Ocular iontophoresis enhances ocular drug delivery by employing a mild electric charge to induce 3 processes: 1.electroporation, i.e. an alteration of ocular tissue structure and pore formation induced by an electric field, 2. electrophoresis and 3. electro-osmosis, i.e. a convective solvent flow through an applied electric potential. This technique can be utilized for anterior segment drug delivery through the trans-corneal route.

EGP-437 using the EyeGate® II Drug Delivery System (EGDS)

EGP-437 using the EyeGate® II Drug Delivery System (EGDS, Eyegate Pharmaceuticals, Inc.) applies ocular iontophoresis to deliver dexamethasone phosphate for the treatment of post cataract surgery inflammation and non-infectious uveitis. In this system, an applicator is placed at the limbus and a generator connected to an electrode is attached to the patient's forehead. The generator creates an electric field inside the applicator, where the drug is loaded, and an opposite charge on the electrode. The difference in charge facilitates the movement of drug molecules through the conjunctiva and sclera. The need for specialised equipment, as well as accurate placement of the applicator on the eye makes it less practical for home use. 80-90% of patients experienced at least one ocular event, with ocular hyperemia and keratitis being the most common, although the incidence of hyperemia appeared to decrease with repeated applications of iontophoresis.[45] A Phase 2 clinical trial was conducted evaluating the safety and efficacy of ocular iontophoretic delivery of dexamethasone phosphate compared to ocular iontophoresis with a placebo in patients planned for cataract surgery (NCT03180255). Treatments were administered on the day prior to cataract surgery. On Day 7, the percentages of patients with anterior chamber cell count of zero in the active and placebo groups were 13% vs 9.1%, respectively, a result that was not clinically significant.[46] With regards to non-infectious anterior uveitis, a Phase 3 trial showed that EGP-437 (NCT02517619) was

indeed clinically efficacious, but it did not achieve non-inferiority compared to prednisolone acetate eyedrops as measured by the proportion of subjects with an anterior chamber cell count of zero at Day 14.[47 48] An imbalance in disease severity, with a greater proportion of subjects in the EGP-437 group having anterior chamber cell scores of 3-4 compared to prednisolone acetate eyedrops (54% vs 41%), and the possible need for an additional iontophoretic treatment were suggested as a possible reasons for the failure to achieve the study endpoint.[49]

IV. Conclusion and perspectives

A summary of the drug delivery technologies for anterior segment inflammation discussed above is given in **Table 1**. Despite its shortcomings, topical steroid eyedrops are still the most widely used treatment for anterior chamber inflammation for the past century. This is set to change in the near future, driven by advances in healthcare delivery, nanomedicine and patient expectations. Corticosteroids and other anti-inflammatory drugs, delivered by the ideal DDS, should provide targeted and sustained drug delivery to inflamed tissue while avoiding off target effects, particularly in the trabecular meshwork which often leads to raised intraocular pressure and glaucoma. The drug delivery system itself should be well tolerated both in terms of the way delivery is performed (non-invasive) and in terms of ocular adverse events. There is great demand, not just for post ocular surgery inflammation, but also for post corneal transplant patients that require long term steroid use. These patients in particular will benefit from an improvement in the therapeutic index of ocular steroids. DDS have the potential for sustained delivery of the lowest dose of steroid non-invasively and without reliance on patient compliance. Pioneering products paving the future for further improvement of anti-inflammatory ocular DDS therapy are Dextenza, Dexycu, INVELTYS and Bromsite which have achieved FDA approval. However, these products are still not widely accepted by patients and the ophthalmic community. Possible reasons for the low adoption rate include, for example, Bromsite and INVELTYS still require self-administration on a twice daily dosing regimen, Dexycu administration requires an invasive intraocular injection into the anterior chamber, and

intracanalicular implants may cause trauma to the tear duct and could be dislodged prematurely. Nanoparticles and ocular iontophoresis form the next wave of DDS that have the potential to replace topical steroids eyedrops as the treatment of choice for anterior segment inflammation. These systems have the advantages of non-invasive (and thus safer) routes of administration by the physician rather than the patient, thereby eliminating patient compliance issues. However, none of the currently available DDS have adequately addressed the issue of off-target effects, specifically the elevation of intraocular pressure by steroids in certain patients. A DDS that can deliver the anti-inflammatory drug directly to inflamed tissue with minimal release of free drug elsewhere is a much needed solution to this problem. With the current relentless pace of ophthalmic drug delivery research, the pursuit of a new standard of treatment may soon be realised.

Table 1: A summary of drug delivery technologies for anterior segment inflammation

Study/Drug name	Drug molecule	Delivery mechanism	Indication	Route of administration	Status
Wong et al[15]	Prednisolone phosphate and triamcinolone acetonide	PEGylated liposomes	Uveitis, post cataract surgery pain and inflammation	Subconjunctival	Phase 1
INVELTYS[23]	Loteprednol etabonate	Mucus Penetrating Particles	Postoperative inflammation and pain following ocular surgery	Topical	FDA approved
RX10045[25 26]	Resolvin E1	nanomicelles	Post cataract surgery ocular inflammation and pain	Topical	Failed Phase 2
Bromfenac Durasite[50]	Bromfenac	Mucoadhesive DDS (synthetic polymer of cross-linked polyacrylic acid)	Post cataract surgery inflammation and pain	Topical	FDA approved

DexaSite[32]	Dexamethasone	Mucoadhesive DDS (DuraSite 2), a synthetic polymer of cross-linked polyacrylic acid and chitosan	Post cataract surgery inflammation and pain	Topical	Phase III
OCS-01	Dexamethasone	Cyclodextrins	Post cataract surgery inflammation and pain	Topical	Phase II
Surodex[51]	Dexamethasone	lactic acid/glycolic acid copolymer	Post cataract surgery inflammation and pain	Intracameral	Phase II
Dexycu[38]	Dexamethasone	Verisome drug delivery technology	Post cataract surgery inflammation and pain	Intracameral	FDA approved
Dextenza[40]	Dexamethasone	Intracanicular implant	Post cataract surgery inflammation and pain	Intracanicular	FDA approved
Nepafenac Punctal Plug Delivery System [41]	Nepafenac	Punctal plug	Post cataract surgery inflammation and pain	Punctal implant	Phase II
DSP-Visulex[44]	Dexamethasone	Ocular drug applicator	Post cataract surgery inflammation and pain	Conjunctival	Phase I/II

EGP-437[46-48]	Dexamethasone	Ocular iontophoresis	Uveitis, Post cataract surgery inflammation and pain	Topical	Phase III (uveitis) Phase II (post cataract surgery)

References

1. McColgin AZ, Heier JS. Control of intraocular inflammation associated with cataract surgery. Curr Opin Ophthalmol 2000;**11**(1):3-6

2. Liu YC, Wilkins M, Kim T, Malyugin B, Mehta JS. Cataracts. Lancet 2017;**390**(10094):600-12 doi: 10.1016/S0140-6736(17)30544-5[published Online First: Epub Date]|.

3. de Smet MD, Taylor SR, Bodaghi B, et al. Understanding uveitis: the impact of research on visual outcomes. Prog Retin Eye Res 2011;**30**(6):452-70 doi: 10.1016/j.preteyeres.2011.06.005[published Online First: Epub Date]|.

4. Schwartz GF, Hollander DA, Williams JM. Evaluation of eye drop administration technique in patients with glaucoma or ocular hypertension. Curr Med Res Opin 2013;**29**(11):1515-22 doi: 10.1185/03007995.2013.833898[published Online First: Epub Date]|.

5. Tatham AJ, Sarodia U, Gatrad F, Awan A. Eye drop instillation technique in patients with glaucoma. Eye (Lond) 2013;**27**(11):1293-8 doi: 10.1038/eye.2013.187[published Online First: Epub Date]|.

6. An JA, Kasner O, Samek DA, Levesque V. Evaluation of eyedrop administration by inexperienced patients after cataract surgery. J Cataract Refract Surg 2014;**40**(11):1857-61 doi: 10.1016/j.jcrs.2014.02.037[published Online First: Epub Date]|.

7. Renfro L, Snow JS. Ocular effects of topical and systemic steroids. Dermatol Clin 1992;**10**(3):505-12

8. Madni A, Sarfraz M, Rehman M, et al. Liposomal drug delivery: a versatile platform for challenging clinical applications. J Pharm Pharm Sci 2014;**17**(3):401-26 doi: 10.18433/j3cp55[published Online First: Epub Date]|.

9. van Rooijen N, van Nieuwmegen R. Liposomes in immunology: multilamellar phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without any immunogenic activity of its own. Immunol Commun 1980;**9**(3):243-56 doi: 10.3109/08820138009065997[published Online First: Epub Date]|.

10. Ahmad I, Allen TM. Antibody-mediated specific binding and cytotoxicity of liposome-entrapped doxorubicin to lung cancer cells in vitro. Cancer Res 1992;**52**(17):4817-20

11. Milla P, Dosio F, Cattel L. PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. Curr Drug Metab 2012;**13**(1):105-19 doi: 10.2174/138920012798356934[published Online First: Epub Date]|.

12. Barza M, Stuart M, Szoka F, Jr. Effect of size and lipid composition on the pharmacokinetics of intravitreal liposomes. Invest Ophthalmol Vis Sci 1987;**28**(5):893-900

13. Gupta SK, Velpandian T, Dhingra N, Jaiswal J. Intravitreal pharmacokinetics of plain and liposome-entrapped fluconazole in rabbit eyes. J Ocul Pharmacol Ther 2000;**16**(6):511-8 doi: 10.1089/jop.2000.16.511[published Online First: Epub Date]|.

14. Joseph RR, Tan DWN, Ramon MRM, et al. Characterization of liposomal carriers for the trans-scleral transport of Ranibizumab. Sci Rep 2017;**7**(1):16803 doi: 10.1038/s41598-017-16791-7[published Online First: Epub Date]|.

15. Wong CW, Czarny B, Metselaar JM, et al. Evaluation of subconjunctival liposomal steroids for the treatment of experimental uveitis. Sci Rep 2018;**8**(1):6604 doi: 10.1038/s41598-018-24545-2[published Online First: Epub Date]|.

16. Lorenz K, Dick B, Jehkul A, Auffahrt GU. Inflammatory response after phacoemulsification treated with 0.5% prednisolone acetate or vehicle. Graefes Arch Clin Exp Ophthalmol 2008;**246**(11):1617-22 doi: 10.1007/s00417-008-0908-2[published Online First: Epub Date]|.

17. Chee Wai Wong EW, Josbert M. Metselaar, Gert Storm, Tina T. Wong. Liposomal drug delivery system for anti-inflammatory treatment after cataract surgery: A Phase I/II clinical trial. In submission.

18. Kim YC, Shin MD, Hackett SF, et al. Gelling hypotonic polymer solution for extended topical drug delivery to the eye. Nat Biomed Eng 2020 doi: 10.1038/s41551-020-00606-8[published Online First: Epub Date]|.

19. Kahraman E, Gungor S, Ozsoy Y. Potential enhancement and targeting strategies of polymeric and lipid-based nanocarriers in dermal drug delivery. Ther Deliv 2017;**8**(11):967-85 doi: 10.4155/tde-2017-0075[published Online First: Epub Date]|.

20. Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev 2009;**61**(2):158-71 doi: 10.1016/j.addr.2008.11.002[published Online First: Epub Date]|.

21. Lai SK, O'Hanlon DE, Harrold S, et al. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. Proc Natl Acad Sci U S A 2007;**104**(5):1482-7 doi: 10.1073/pnas.0608611104[published Online First: Epub Date]|.

22. Schopf L, Enlow E, Popov A, Bourassa J, Chen H. Ocular Pharmacokinetics of a Novel Loteprednol Etabonate 0.4% Ophthalmic Formulation. Ophthalmol Ther 2014;**3**(1-2):63-72 doi: 10.1007/s40123-014-0021-z[published Online First: Epub Date]|.

23. Kim T, Sall K, Holland EJ, Brazzell RK, Coultas S, Gupta PK. Safety and efficacy of twice daily administration of KPI-121 1% for ocular inflammation and pain following cataract surgery. Clin Ophthalmol 2019;**13**:69-86 doi: 10.2147/OPTH.S185800[published Online First: Epub Date]|.

24. Mandal A, Bisht R, Rupenthal ID, Mitra AK. Polymeric micelles for ocular drug delivery: From structural frameworks to recent preclinical studies. J Control Release 2017;**248**:96-116 doi: 10.1016/j.jconrel.2017.01.012[published Online First: Epub Date]|.

25. Cholkar K, Gilger BC, Mitra AK. Topical delivery of aqueous micellar resolvin E1 analog (RX-10045). Int J Pharm 2016;**498**(1-2):326-34 doi: 10.1016/j.ijpharm.2015.12.037[published Online First: Epub Date]|.

26. Efficacy and Safety of RX-10045 Ophthalmic Solution for Ocular Inflammation and Pain in Cataract Surgery.

27. Cholkar K, Trinh HM, Vadlapudi AD, Wang Z, Pal D, Mitra AK. Interaction Studies of Resolvin E1 Analog (RX-10045) with Efflux Transporters. J Ocul Pharmacol Ther 2015;**31**(4):248-55 doi: 10.1089/jop.2014.0144[published Online First: Epub Date]|.

28. Goldberg DF, Malhotra RP, Schechter BA, Justice A, Weiss SL, Sheppard JD. A Phase 3, Randomized, Double-Masked Study of OTX-101 Ophthalmic Solution 0.09% in the Treatment of Dry Eye Disease. Ophthalmology 2019;**126**(9):1230-37 doi: 10.1016/j.ophtha.2019.03.050[published Online First: Epub Date]|.

29. Weiss SL, Kramer WG. Ocular Distribution of Cyclosporine Following Topical Administration of OTX-101 in New Zealand White Rabbits. J Ocul Pharmacol Ther 2019;**35**(7):395-402 doi: 10.1089/jop.2018.0106[published Online First: Epub Date]|.

30. Hosseini K, Walters T, DaVanzo R, Lindstrom RL. A randomized double-masked study to compare the ocular safety, tolerability, and efficacy of bromfenac 0.075% compared with vehicle in cataract surgery subjects. Clin Ophthalmol 2016;**10**:2311-17 doi: 10.2147/OPTH.S120428[published Online First: Epub Date]|.

31. Shafiee A, Bowman LM, Hou E, Hosseini K. Aqueous humor penetration of ketorolac formulated in DuraSite or DuraSite 2 delivery systems compared to Acular LS in rabbits. J Ocul Pharmacol Ther 2013;**29**(9):812-6 doi: 10.1089/jop.2013.0016[published Online First: Epub Date]|.

32. Study to Evaluate ISV-305 Compared to Vehicle for Treatment of Inflammation and Pain Associated With Cataract Surgery (ISV-305).

33. OCS-01 in Treating Inflammation and Pain in Post-cataract Patients (SKYGGN).

34. oculis-reports-positive-top-line-data-from-skyggn-phase-2-trial-with-ocs-01. Secondary oculis-reports-positive-top-line-data-from-skyggn-phase-2-trial-with-ocs-01. https://oculis.com/2020/05/18/oculis-reports-positive-top-line-data-from-skyggn-phase-2-trial-with-ocs-01/.

35. Chang DF, Wong V. Two clinical trials of an intraocular steroid delivery system for cataract surgery. Trans Am Ophthalmol Soc 1999;**97**:261-74; discussion 74-9

36. Tan DT, Chee SP, Lim L, Lim AS. Randomized clinical trial of a new dexamethasone delivery system (Surodex) for treatment of post-cataract surgery inflammation. Ophthalmology 1999;**106**(2):223-31 doi: 10.1016/S0161-6420(99)90060-X[published Online First: Epub Date]|.

37. Donnenfeld E, Holland E. Dexamethasone Intracameral Drug-Delivery Suspension for Inflammation Associated with Cataract Surgery: A Randomized, Placebo-Controlled, Phase III Trial. Ophthalmology 2018;**125**(6):799-806 doi: 10.1016/j.ophtha.2017.12.029[published Online First: Epub Date]|.

38. Donnenfeld ED, Solomon KD, Matossian C. Safety of IBI-10090 for inflammation associated with cataract surgery: Phase 3 multicenter study. J Cataract Refract Surg 2018;**44**(10):1236-46 doi: 10.1016/j.jcrs.2018.07.015[published Online First: Epub Date]|.

39. Blizzard C, Desai A, Driscoll A. Pharmacokinetic Studies of Sustained-Release Depot of Dexamethasone in Beagle Dogs. J Ocul Pharmacol Ther 2016;**32**(9):595-600 doi: 10.1089/jop.2016.0025[published Online First: Epub Date]|.

40. Tyson SL, Bafna S, Gira JP, et al. Multicenter randomized phase 3 study of a sustained-release intracanalicular dexamethasone insert for treatment of ocular inflammation and pain after cataract surgery. J Cataract Refract Surg 2019;**45**(2):204-12 doi: 10.1016/j.jcrs.2018.09.023[published Online First: Epub Date]|.

41. Safety/Efficacy of Nepafenac Punctal Plug Delivery System Compared to Placebo to Control Ocular Pain/Inflammation After Cataract NCT03496467.

42. Papangkorn K, Prendergast E, Higuchi JW, Brar B, Higuchi WI. Noninvasive Ocular Drug Delivery System of Dexamethasone Sodium Phosphate in the Treatment of Experimental Uveitis Rabbit. J Ocul Pharmacol Ther 2017;**33**(10):753-62 doi: 10.1089/jop.2017.0053[published Online First: Epub Date]|.

43. Papangkorn K, Higuchi JW, Brar B, Higuchi WI. Ocular Drug Distribution and Safety of a Noninvasive Ocular Drug Delivery System of Dexamethasone Sodium Phosphate in Rabbit. J Ocul Pharmacol Ther 2018;**34**(4):325-34 doi: 10.1089/jop.2017.0093[published Online First: Epub Date]|.

44. Papangkorn K, Truett KR, Vitale AT, et al. Novel Dexamethasone Sodium Phosphate Treatment (DSP-Visulex) for Noninfectious Anterior Uveitis: A Randomized Phase I/II Clinical Trial. Curr Eye Res 2019;**44**(2):185-93 doi: 10.1080/02713683.2018.1540707[published Online First: Epub Date]|.

45. Patane MA, Cohen A, From S, Torkildsen G, Welch D, Ousler GW, 3rd. Ocular iontophoresis of EGP-437 (dexamethasone phosphate) in dry eye patients: results of a randomized clinical trial. Clin Ophthalmol 2011;**5**:633-43 doi: 10.2147/OPTH.S19349[published Online First: Epub Date]|.

46. Safety and Efficacy of Iontophoretic Dexamethasone Phosphate Delivery Prior to Implantation of a Posterior Chamber IOL.

47. http://www.eyegatepharma.com/press-release/eyegate-announces-top-line-results-for-phase-3-trial-of-egp-437-in-anterior-uveitis/.

48. Safety and Efficacy Study of Iontophoretic Dexamethasone Phosphate Ophthalmic Solution to Treat Non-Infectious Anterior Segment Uveitis.

49. Sheppard J, Garg S, Lievens C, et al. Iontophoretic Dexamethasone Phosphate Compared to Topical Prednisolone Acetate 1% for Noninfectious Anterior Segment Uveitis. Am J Ophthalmol 2020;**211**:76-86 doi: 10.1016/j.ajo.2019.10.032[published Online First: Epub Date]|.

50. Trattler W, Hosseini K. Twice-Daily vs. Once-Daily Dosing with 0.075% Bromfenac in DuraSite: Outcomes from a 14-Day Phase 2 Study. Ophthalmol Ther 2017;**6**(2):277-84 doi: 10.1007/s40123-017-0102-x[published Online First: Epub Date]|.

51. Tan DT, Chee SP, Lim L, Theng J, Van Ede M. Randomized clinical trial of Surodex steroid drug delivery system for cataract surgery: anterior versus posterior placement of two Surodex in the eye. Ophthalmology 2001;**108**(12):2172-81

3

Advances in posterior segment drug delivery for the treatment of exudative age-related macular degeneration and diabetic macular edema

Chee Wai Wong[1,2,3]
Tina T. Wong[1,2,3*]

1. Singapore National Eye Centre (SNEC), 11 Third Hospital Avenue, Singapore 168751
2. Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore 168751
3. Duke-NUS Graduate Medical School, 8 College Rd, Singapore 169857

Br J Ophthalmol. 2019 Oct;103(10):1356-1360.

Abstract

Anti-vascular endothelial growth factors and corticosteroids are used to treat a myriad of retinal conditions, including exudative age-related macular degeneration (AMD) and diabetic macular edema (DME), diabetic retinopathy. Although effective, long term efficacy is limited by the need for frequent and invasive intravitreal injections. Several formulations of intraocular sustained delivery corticosteroid implants have received FDA approval for the treatment of diabetic macular edema, but these implants still require intravitreal administration. The quest for sustained action therapeutics that can be delivered to target tissue in the least invasive manner is a treacherous endeavour that has ended in premature failure for several technologies in Phase 2 or 3 trials. Nevertheless, there have been notable successes, and more are on the horizon: port delivery systems for the treatment of exudative AMD have entered Phase 3 trials and a wide array of preclinical studies have demonstrated the potential for nanoparticles, such as liposomes, dendrimers, polymeric micelles, and cell penetrating peptides to deliver therapeutics into the posterior segment via minimally invasive routes. In this review, we discuss the challenges posed by ocular barriers for drug penetration and present the recent advancements of the most pertinent drug delivery platforms with a focus on the treatment of exudative AMD and DME.

Introduction

Anti-vascular endothelial growth factors (anti-VEGF) and corticosteroids are the most common posterior segment therapeutics for the treatment of retinal conditions including exudative age related macular degeneration (AMD), myopic choroidal neovascularization, diabetic macular edema (DME), diabetic retinopathy and retinal vein occlusion related cystoid macular edema. Though effective, there are several drawbacks to these therapies. First, anti-VEGF molecules are large and highly hydrophilic, making penetration through ocular barriers problematic. Thus, invasive intravitreal injections are required to deliver sufficient drug concentrations to the retina. Intravitreal injections are associated with risks of infection, retinal detachment, glaucoma and lens damage. Second, they have short ocular half-lives relative to the duration of disease activity, thus necessitating frequent and repeated intravitreal injections. Consequently, the need for frequent injections increases the cumulative risks of injection related complications and has led to a tremendous treatment burden as well as escalating costs. Long-term follow up of patients who have exited large AMD treatment trials illustrate the challenges of sustaining frequent and costly intravitreal injections, with most patients losing vision gained from the first 2 years of anti-VEGF therapy.[1 2]

Driven by the shortcomings of invasive and inefficient posterior segment drug delivery, there is a huge impetus for the research and development of non or minimally invasive, sustained action therapeutics for these diseases. Several formulations of intraocular corticosteroid implants are currently approved by the FDA to treat diabetic macular edema. However, these implants still require administration via intravitreal injection. To circumvent ocular barriers to posterior segment drug delivery and reduce the need for repeated injections, several drug delivery mechanisms have been developed. Among these, nanoparticles such as liposomes, dendrimers, polymeric micelles, conjugation of therapeutic payload with cell penetrating peptides and port delivery systems have shown the most promise. In this review, we describe the challenges posed by ocular barriers to drug

penetration and present the recent advancements in the most pertinent drug delivery platforms with a focus on the treatment of exudative AMD and DME.

Barriers to ocular drug delivery

Ocular drug delivery in a non-invasive manner remains a unique challenge due to complex barriers that limit drug bioavailability by the topical and systemic routes. The main penetration barriers to the posterior segment are the tear film, conjunctiva, cornea, sclera, blood aqueous barrier and blood retinal barrier.

Tear film and conjunctival sac

Bioavailability of topically administered eyedrops is poor due to limited capacity of the conjunctival sac (25µl), short precorneal drug residence time and drainage of drug via the nasolacrimal duct. It has been estimated that the ocular absorption of topically applied drugs is less than 5%.[3] Following topical application, a significant increase in tearing dilutes the administered dose, decreasing drug concentration and diminished drug absorption. Increased lacrimation also leads to rapid clearance from the precorneal tear film and spillage of the administered drug further reduces precorneal drug residence time.[4]

Cornea

The cornea is an efficient multilayered penetration barrier consisting of the epithelium, stroma and endothelium. Of these, the epithelium is the main barrier to drug penetration. Intercellular tight junctions, known as zonula occludens, within the lipophilic epithelium prevents diffusion of large molecules with only small, lipophilic molecules able to traverse the epithelium transcellularly. The stroma is highly hydrophilic in nature and only allows the diffusion of hydrophilic molecules up to

500 kDa of size, while restricting the entry of most lipophilic drugs.[5] Lastly, the corneal endothelium restricts diffusion to small molecules up to about 20 nm.[6]

Conjunctiva and sclera

The conjunctiva is less of a barrier than the cornea because of a lower number of intercellular tight junctions, but the vascularity of both the conjunctival stroma and the corneoscleral limbus increases the clearance and systemic absorption of drug molecules.[7] Subconjunctival or subtenon's injections bypass the conjunctival barrier and places the drug molecules in direct contact with the sclera. The sclera, with its large surface area (95% of total globe surface) and high permeability makes the trans-scleral route an ideal minimally invasive route for drug delivery to the posterior segment. Indeed, studies have shown that vitreal concentrations of drug molecules are highest following subconjunctival injection compared to oral administration or peribulbar injection.[8 9] Scleral permeability is facilitated by scleral water channels/pores (ranging from 30 to 300 nm in size) thorough which passive diffusion of drug molecules can occur.[10]

Vitreous

The vitreous humor is composed of water (98– 99%) with only a few solid components such as collagen and glycosaminoglycans. Drug molecules move through the vitreous via 2 main processes: diffusion and convection. The vitreous is a loose structure that poses no diffusional resistance to movement of small (<500nm) and anionic particles but restricts the movement of larger cationic particles.[11] Liquefaction of the vitreous with ageing, and in the case of previous vitrectomy, complete loss of the collagenous vitreous gel structure, enhances convective flow within the vitreous cavity and leads to a shorter drug residence time.[12] Smaller sized molecules have also been shown to have a longer elimination half-life compared to larger ones, although the reason for this is still unclear.[13]

Blood retinal barrier

The blood retinal barrier (BRB) can be divided into the inner and outer BRB. The inner BRB is closest to the vitreous and is composed of tight junctions between retinal endothelial blood vessels. The outer BRB is closest to the choroid and is formed by tight junctions between retinal pigment epithelium (RPE) cells.[14] These barriers restrict passive penetration of drug molecules into the retina via both the systemic and transscleral routes.

Drug delivery for the posterior segment (table 1)

Intraocular implants

Iluvien (Alimera Sciences, Alpharetta, USA) is a non-bio erodible implant consisting of a cylindrical polyimide tube delivering fluocinolone acetonide for the treatment of diabetic macular edema. It is inserted via a 25 gauge needle as an office based procedure, with therapeutic effect lasting up to 36 months. The implant is inert and may be retained in the eye. In the FAME study, a randomized controlled trial of Iluvien for the treatment of diabetic macular edema, about 38% of patients developed raised IOP that could be managed with glaucoma medications and 4.8% further required glaucoma surgery to control the raised IOP.[15 16] However, besides the inconvenience of obstructing vision when the implant moves into the visual axis, the long-term effects of non-biodegradable implants on ocular structures such as the lens, zonules and retina is unknown.

To circumvent the long-term risks of non-biodegradable implants, the bioerodable, extended-release dexamethasone delivery system (**Ozurdex**, Allergan, Irvine, CA) was developed. Ozurdex is approved by the FDA for the treatment of diabetic macular edema, macular edema secondary to retinal vein occlusion and non-infectious posterior uveitis. It is injected into the posterior segment with a 25-gauge needle as an office-based procedure. Each implant can last 3-4 months. In a 3 year randomized controlled trial evaluating the use of ozurdex for diabetic macular edema, one third of patients who received ozurdex developed raised IOP but none of the patients required removal of the implant to control IOP and only 0.3% required glaucoma surgery.[17]

Topical eyedrops

Drug potency and effective dose are crucial for its suitability to be delivered topically. Retinal bioavailability is low after topical administration (≪ 0.1%)[8], but the concentrations needed for pharmacological activity are compound and size dependent. Topical administration may be suitable only for highly potent compounds that are active at low concentrations, preferably in the nanomolar or picomolar range in size. Accordingly, several topical anti-angiogenic eyedrops have been developed but most have failed to demonstrate benefit over intravitreal anti-VEGF in clinical trials. These therapies are described briefly in this section.

Pazopanib (GlaxoSmithKline, Brentford, UK) is a multi-targeted tyrosine kinase inhibitor that inhibits tumor growth factors such as stem cell growth factor (c-KIT), fibroblast growth factor receptor (FGFR), PDGFR and VEGFR 1-3. In a large Phase 3 clinical trial, pazopanib eye drops administered daily in conjunction with monthly or as needed ranibizumab did not provide additional therapeutic benefit and did not reduce the number of as needed ranibizumab injections by the prespecified ≥50% criteria.[18] Similarly, **Regorafenib** (Bayer Pharma, Leverkusen, Germany), another multi-kinase inhibitor that has been evaluated as an eye drop therapy for exudative AMD was unable to achieve similar visual acuity gains to established anti-VEGF therapies and was terminated after completion of Phase 2a.

Squalamine is a small molecule inhibitor of multiple growth factors, including VEGF, PDGF and basic fibroblast growth factor (bFGF). In the MAKO trial, a Phase 3 trial that included 237 patients with neovascular AMD, squalamine failed to add any benefit when used in combination with ranibizumab, compared to ranibizumab therapy.[19]

Pan-90806 (PanOptica, Bernardsville, NJ, USA) is a topically administered inhibitor of VEGF receptor 2, fibroblast growth factor 1–3, tyrosine kinase endothelial receptor 2, and other proangiogenic factors.[20] Initial results from the Phase1/2 study are promising, with 45-50% of

treated patients with a positive response with regards to vision, lesion morphology and retinal thickness.

Nanoparticles

Liposomes

Liposomes range in size from 0.01 to 10 μm and consist of a single outer lipid bilayer or multiple interwoven lipid bilayers (multilamellar) composed primarily of phospholipids and cholesterol, and an aqueous internal compartment. Liposomes have several advantages that make them suitable as a drug delivery mechanism, including biocompatibility and biodegradability, amphipathic nature allowing incorporation of both hydrophobic and hydrophilic drug molecules, surface modification to enhance solubility and specificity, and the ability to increase half-life of the encapsulated drug by 7-11 times.[21-23] [24] [25-27] Because of their amphipathic nature, liposomes can be utilised as carriers for both hydrophilic anti-VEGF agents (<10nm) and hydrophobic corticosteroids. Their small size and modifiable surface charge facilitates their passage through the vitreous to reach the therapeutic target sites in the retina.

To date, **Visudyne (Verteporfin)** remains the only FDA approved liposomal light-activated formulation used for the treatment of exudative AMD.[28] Visudyne is a hydrophobic molecule encapsulated in liposome with high affinity for low density lipoprotein receptors on the endothelial cells of choroidal neovascular membranes. Otherwise inert, it becomes an efficient generator of free radicals when activated by non-thermal laser light at 689nm. Visudyne has largely been superseded by anti-VEGF therapy for the treatment of neovascular AMD.[29]

Liposome encapsulated anti-VEGF agents have shown promise both in terms of efficient delivery through non-invasive or minimally invasive routes or by increasing drug residency time within the vitreous. Annexin A5 associated liposomes loaded with bevacizumab applied topically once a day for

5 days in New Zealand white rabbits achieved significantly higher concentrations of bevacizumab in the vitreous and retina than an equivalent topical dose of unencapsulated avastin.[30] These liposomes were able to undergo transcytosis across the cornea epithelia and possibly the conjunctiva epithelia as well, allowing them to bypass the corneal barrier. In experimental models, ex vivo transport of liposome encapsulated ranibizumab across sclera (simulating a subconjunctival injection) occurred in a linear manner for seven days, suggesting that the sclera allows diffusion of liposomal-formulated ranibizumab and raises the possibility that subconjunctival injections could serve as long-acting depots for anti-VEGF therapy.[27] In rabbits, intravitreal injection of liposomal bevacizumab was shown to produce 5 times higher intravitreal concentration of bevacizumab at day 42 compared to free bevacizumab.[31]

The potential of liposomal steroids for efficient vitreoretinal drug delivery have been studied. A topical formulation of triamcinolone acetonide (TA) loaded liposomes were administered in New Zealand white rabbits and demonstrated that liposome encapsulated TA could reach the vitreous and retina efficiently.[32] Liposomal TA has also been observed to be able to diffuse across rabbit sclera ex vivo, with strong tissue binding providing a drug depot for sustained release.[33]

Dendrimers

Dendrimers are a group of polymeric nanostructures that have been extensively investigated for ocular drug delivery. Their enhanced aqueous solubility, smaller size (1-100nm) and large variety of surface functional groups that can be modified for targeted delivery makes them ideal as a versatile and biocompatible drug delivery mechanism.[34] As with liposomes, the size and charge of dendrimers grants them smooth passage through the vitreous, and they are capable of delivering both hydrophobic and hydrophilic drug molecules.

In a laser induced CNV rat model, a lipophilic amino acid dendrimer-VEGF oligonucleotide (ODN-1) conjugate significantly inhibited the development of CNV for 4-6 months, while injection of ODN-1 alone was unable to inhibit CNV activity from month 2 onwards. The dendrimer ODN-1 conjugates

were also shown to penetrate all layers of the retina to the retinal pigment epithelium.[35] The dendrimer in this instance provided a high degree of protection against nucleases and enhanced delivery of the ODN-1 gene into retinal tissues, thereby prolonging its effective lifespan.

Yavuz et al evaluated a dendrimer-dexamethasone conjugate in ex vivo transport studies across rabbit cornea and sclera-choroid-retinal pigment epithelial tissues and showed that the dexamethasone conjugates exhibited higher drug transport levels than free dexamethasone. In vivo ocular distribution of dendrimer-dexamethasone conjugates following both topical and subconjunctival application into eyes of Sprague– Dawley rats showed that ocular bioavailability was enhanced compared to free dexamethasone via both routes of administration.[36]

Polymeric micelles

Polymeric micelles (10– 200 nm) are self-assembling, amphiphilic molecules or block copolymers with a hydrophobic core and a hydrophilic shell. They increase the solubility of hydrophobic drugs by encapsulating them within the hydrophobic core, resulting in clear aqueous formulations. Polymeric micelles are thus excellent for the encapsulation of hydrophobic corticosteroids. Their small size and hydrophilic nature enable efficient penetration of ocular barriers including the cornea, sclera and vitreous. In addition, core cross-linked polymeric micelles allow increased drug retention and tailored release kinetics and have received much attention for ocular drug delivery.[37] Dexamethasone encapsulated polymeric nanomicelles (25-30nm) have demonstrated significantly enhanced permeability, by 2.5 times compared to free dexamethasone, in an ex vivo study of transport across rabbit sclera.[38] The release kinetics of triamcinolone acetonide was significantly improved by their encapsulation within micelles and in combination with a reverse thermal gel system, was projected to sustain release for a year based on in vitro testing.[39]

Port Delivery Systems

A refillable ranibizumab port delivery system has been developed to reduce the need for repeated intravitreal anti-VEGF injections. The preloaded implant is surgically implanted beneath the conjunctiva through a 3.2 mm scleral incision over the pars plana, with a reservoir tip that can be accessed easily and refilled through the conjunctiva as needed. This device essentially replaces regular intravitreal injections with a single surgical implantation of the port delivery system. In between refills, the device continuously releases ranibizumab into the vitreous. Following a successful Phase 1 trial in patients with neovascular age related macular degeneration, a multicentre, randomized, treatment-control, Phase 2 LADDER trial was conducted and recently announced positive topline results, with the majority of patients requiring a refill only after the 6 months and achieving similar visual outcomes as monthly injections of ranibizumab.[40]

Microelectromechanical system (MEMS) technology is a miniaturized system that is currently used in insulin pumps to deliver drug to tissues. The Posterior MicroPump Drug Delivery System (PMP, Replenish Inc., Pasadena, CA, USA) prefilled with ranibizumab has been tested in a first in man trial of 11 patients with diabetic macular edema. The PMP was implanted in the subconjunctival space with an intraocular cannula inserted through the pars plana, and delivers a programmed microdose via wireless control. In 7 patients, the PMP delivered the programmed dose of ranibizumab while the remaining 4 received a lower than target dose.[41] The device was well tolerated with no serious adverse events reported during the follow up period.

Cell Penetrating Peptides (CPPs)

CPPs are a family of various peptides, typically sequences of 5-40 amino acids (aa), also known as "Trojan horse peptides" for their ability to deliver a wide range of cargo across bio-barriers via energy-dependent or energy-independent mechanisms with no interaction with specific receptors.[42] [43] CPPs can enhance the cellular internalization of covalently or noncovalently conjugated

therapeutic cargo and thus have received considerable interest as a means of drug delivery.[42] De Cogan et al recently reported the possibility of topically delivering anti-VEGF to the posterior segment by conjugating bevacizumab or ranibizumab to a CPP (FAM (5-carboxyfluorescein) polyarginine R6). They reported rapid diffusion of topically administered CPP + anti-VEGF into the anterior chamber (within 6 minutes), reaching maximum tissue concentration in the vitreous and retina after 45 minutes in the rat model, and demonstrated the same capability in an ex vivo model using the porcine eye. In a mouse model of laser induced choroidal neovascularization (CNV), twice daily CPP + anti-VEGF eye drops was comparable to a single intravitreal injection of anti-VEGF in reducing CNV area.[44] These are promising results and CPPs conjugated to other anti-angiogenic agents such as endostatin,[45] KV11[46] and tyrosine kinase inhibitors (pazopanib)[47] have demonstrated similar efficacy with noninvasive or minimally invasive routes of administration in preclinical models.

One notable drawback of CPPs is their lack of cell and tissue specificity, which could potentially lead to a wider drug distribution and intracellular accumulation than is desired for the specific indication. Another downside is their rapid degradation by both extracellular and intracellular proteases, releasing the cargo before they reach their target sites and thus reducing their efficacy. It is hoped that ongoing research will be able to address both the specificity and stability concerns of CPPs.[48]

Table 1: A summary of posterior segment drug delivery technologies

Study/Drug name	**Drug molecule**	**Delivery mechanism**	**Indication**	**Proposed route of administration**	**Status**
Illuvien[15]	Fluocinolone acetonide	Non bioerodable polymer implant	DME	Intravitreal injection	FDA approved
Ozurdex[17]	Dexamethasone	Bioerodable polymer implant	DME	Intravitreal injection	FDA approved
Pazopanib[18]	Tyrosine kinase inhibitor	eyedrops	Exudative AMD	Topical	Failed Phase 2a
Squalamine[19]	VEGF/PDGF/bFGF inhibitor	Eyedrops	Exudative AMD	Topical	Failed Phase 3
Pan-90806[20]	VEGF/FGF/tyrosine kinase inhibitor	Eyedrops	Exudative AMD	Topical	Phase 1/2
Visudyne[28]	Verteporfin	Liposome	Exudative AMD	Intravenous	FDA approved
Davis BM et al, 2014[30]	Bevacizumab	Liposome	Exudative AMD	Topical	Preclinical

Abrishami M et al, 2009[31]	Bevacizumab	Liposome	Exudative AMD	Intravitreal	Preclinical
Joseph RR et al, 2017[27]	Ranibizumab	Liposome	Exudative AMD	Subconjunctival	Preclinical
Altamirano-Vallejo JC et al, 2018[32]	Triamcinolone acetonide	Liposome	DME	Topical	Preclinical
Araujo J et al, 2012[33]	Triamcinolone acetonide	Liposome	DME	Subconjunctival	Preclinical
Marano RJ et al, 2005[35]	ODN-1	Dendrimer	Exudative AMD	Intravitreal	Preclinical
Yavuz B et al, 2017[36]	Dexamethasone	Dendrimer	DME	Topical and subconjunctival	Preclinical
Vaishya RD et al, 2014[38]	Dexamethasone	Polymeric micelles	Intermediate and posterior uveitis	Subconjunctival	Preclinical
Famili A et al, 2014[39]	Triamcinolone acetonide	Polymeric micelles + reverse thermal gel system	DME	Intravitreal	Preclinical
PDS[40]	Ranibizumab	Refillable port delivery system	Exudative AMD	Trans-scleral implantation	Phase 3 trials
PMP[41]	Ranibizumab	Refillable micropump	DME	Trans-scleral implantation	Phase 1

De Cogan et al, 2017[44]	Bevacizumab/Ranibizumab	Cell penetrating peptides	Exudative AMD	Topical	Preclinical
Zhang X et al, 2015[45]	Endostatin	Cell penetrating peptides	Exudative AMD	Topical	Preclinical
Chen C et al, 2017[46]	KV11	Cell penetrating peptides	Exudative AMD	Topical/retrobulbar injection	Preclinical
Suda K et al, 2017[47]	Tyrosine kinase inhibitor	Cell penetrating peptides	Exudative AMD	Topical	Preclinical

Abbreviations: AMD, age related macular degeneration; DME, diabetic macular edema; VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; ODN-1, VEGF oligonucleotide.

Conclusions and future directions

Anti-VEGF therapy has transformed the treatment of exudative AMD and DME, but the fight against blindness from these conditions is far from over. Long-term studies have starkly revealed the shortcomings of current treatment. The next major step for the field is to develop sustained action therapeutics that can be delivered to target tissue in the least invasive manner. This is an arduous task and many promising technologies have met with premature failure in Phase 2 or 3 trials. Nevertheless, there have been notable successes, and more are on the horizon. It is a testimony that better, more clinically effective therapies and sustained delivery systems remain a focused area in the advancement of clinical treatment of AMD and DME. Thus, it is imperative for pharmaceutical scientists, ophthalmologists and industry to collaborate and expedite the clinical translation of such promising therapies.

References

1. Comparison of Age-related Macular Degeneration Treatments Trials Research G, Maguire MG, Martin DF, et al. Five-Year Outcomes with Anti-Vascular Endothelial Growth Factor Treatment of Neovascular Age-Related Macular Degeneration: The Comparison of Age-Related Macular Degeneration Treatments Trials. Ophthalmology 2016;**123**(8):1751-61 doi: 10.1016/j.ophtha.2016.03.045[published Online First: Epub Date]|.

2. Rofagha S, Bhisitkul RB, Boyer DS, Sadda SR, Zhang K, Group S-US. Seven-year outcomes in ranibizumab-treated patients in ANCHOR, MARINA, and HORIZON: a multicenter cohort study (SEVEN-UP). Ophthalmology 2013;**120**(11):2292-9 doi: 10.1016/j.ophtha.2013.03.046[published Online First: Epub Date]|.

3. Urtti A, Salminen L. Minimizing systemic absorption of topically administered ophthalmic drugs. Surv Ophthalmol 1993;**37**(6):435-56

4. Lee VH, Robinson JR. Topical ocular drug delivery: recent developments and future challenges. J Ocul Pharmacol 1986;**2**(1):67-108

5. Rajapakshal A, Fink M, Todd BA. Size-Dependent Diffusion of Dextrans in Excised Porcine Corneal Stroma. Mol Cell Biomech 2015;**12**(3):215-30

6. Yellepeddi VK, Palakurthi S. Recent Advances in Topical Ocular Drug Delivery. J Ocul Pharmacol Ther 2016;**32**(2):67-82 doi: 10.1089/jop.2015.0047[published Online First: Epub Date]|.

7. Barar J, Javadzadeh AR, Omidi Y. Ocular novel drug delivery: impacts of membranes and barriers. Expert Opin Drug Deliv 2008;**5**(5):567-81 doi: 10.1517/17425247.5.5.567[published Online First: Epub Date]|.

8. Weijtens O, Feron EJ, Schoemaker RC, et al. High concentration of dexamethasone in aqueous and vitreous after subconjunctival injection. Am J Ophthalmol 1999;**128**(2):192-7

9. Weijtens O, Schoemaker RC, Lentjes EG, Romijn FP, Cohen AF, van Meurs JC. Dexamethasone concentration in the subretinal fluid after a subconjunctival injection, a peribulbar injection, or an oral dose. Ophthalmology 2000;**107**(10):1932-8

10. Prausnitz MR, Noonan JS. Permeability of cornea, sclera, and conjunctiva: a literature analysis for drug delivery to the eye. J Pharm Sci 1998;**87**(12):1479-88

11. Del Amo EM, Rimpela AK, Heikkinen E, et al. Pharmacokinetic aspects of retinal drug delivery. Prog Retin Eye Res 2017;**57**:134-85 doi: 10.1016/j.preteyeres.2016.12.001[published Online First: Epub Date]|.

12. Mains J, Wilson CG. The vitreous humor as a barrier to nanoparticle distribution. J Ocul Pharmacol Ther 2013;**29**(2):143-50 doi: 10.1089/jop.2012.0138[published Online First: Epub Date]|.

13. Sakurai E, Ozeki H, Kunou N, Ogura Y. Effect of particle size of polymeric nanospheres on intravitreal kinetics. Ophthalmic Res 2001;**33**(1):31-6 doi: 10.1159/000055638[published Online First: Epub Date]|.

14. Campbell M, Humphries P. The blood-retina barrier: tight junctions and barrier modulation. Adv Exp Med Biol 2012;**763**:70-84

15. Saedon H, Anand A, Yang YC. Clinical utility of intravitreal fluocinolone acetonide (Iluvien((R))) implant in the management of patients with chronic diabetic macular edema: a review of the current literature. Clin Ophthalmol 2017;**11**:583-90 doi: 10.2147/OPTH.S131165[published Online First: Epub Date]|.

16. Campochiaro PA, Brown DM, Pearson A, et al. Sustained delivery fluocinolone acetonide vitreous inserts provide benefit for at least 3 years in patients with diabetic macular edema. Ophthalmology 2012;**119**(10):2125-32 doi: 10.1016/j.ophtha.2012.04.030[published Online First: Epub Date]|.

17. Boyer DS, Yoon YH, Belfort R, Jr., et al. Three-year, randomized, sham-controlled trial of dexamethasone intravitreal implant in patients with diabetic macular edema. Ophthalmology 2014;**121**(10):1904-14 doi: 10.1016/j.ophtha.2014.04.024[published Online First: Epub Date]|.

18. Csaky KG, Dugel PU, Pierce AJ, et al. Clinical evaluation of pazopanib eye drops versus ranibizumab intravitreal injections in subjects with neovascular age-related macular degeneration. Ophthalmology 2015;**122**(3):579-88 doi: 10.1016/j.ophtha.2014.09.036[published Online First: Epub Date]|.

19. https://globenewswire.com/news-release/2018/01/05/1284092/0/en/Ohr-Pharmaceutical-Announces-Efficacy-Results-from-the-MAKO-Study-in-Wet-AMD.html.

20. Tolentino MJ, Dennrick A, John E, Tolentino MS. Drugs in Phase II clinical trials for the treatment of age-related macular degeneration. Expert Opin Investig Drugs 2015;**24**(2):183-99 doi: 10.1517/13543784.2015.961601[published Online First: Epub Date]|.

21. Madni A, Sarfraz M, Rehman M, et al. Liposomal drug delivery: a versatile platform for challenging clinical applications. J Pharm Pharm Sci 2014;**17**(3):401-26

22. van Rooijen N, van Nieuwmegen R. Liposomes in immunology: multilamellar phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without any immunogenic activity of its own. Immunol Commun 1980;**9**(3):243-56

23. Ahmad I, Allen TM. Antibody-mediated specific binding and cytotoxicity of liposome-entrapped doxorubicin to lung cancer cells in vitro. Cancer Res 1992;**52**(17):4817-20

24. Milla P, Dosio F, Cattel L. PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. Curr Drug Metab 2012;**13**(1):105-19

25. Barza M, Stuart M, Szoka F, Jr. Effect of size and lipid composition on the pharmacokinetics of intravitreal liposomes. Invest Ophthalmol Vis Sci 1987;**28**(5):893-900

26. Gupta SK, Velpandian T, Dhingra N, Jaiswal J. Intravitreal pharmacokinetics of plain and liposome-entrapped fluconazole in rabbit eyes. J Ocul Pharmacol Ther 2000;**16**(6):511-8 doi: 10.1089/jop.2000.16.511[published Online First: Epub Date]|.

27. Joseph RR, Tan DWN, Ramon MRM, et al. Characterization of liposomal carriers for the trans-scleral transport of Ranibizumab. Sci Rep 2017;**7**(1):16803 doi: 10.1038/s41598-017-16791-7[published Online First: Epub Date]|.

28. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials--TAP report. Treatment of age-related macular degeneration with photodynamic therapy (TAP) Study Group. Arch Ophthalmol 1999;**117**(10):1329-45

29. Brown DM, Michels M, Kaiser PK, et al. Ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degeneration: Two-year results of the ANCHOR study. Ophthalmology 2009;**116**(1):57-65 e5 doi: 10.1016/j.ophtha.2008.10.018[published Online First: Epub Date]|.

30. Davis BM, Normando EM, Guo L, et al. Topical delivery of Avastin to the posterior segment of the eye in vivo using annexin A5-associated liposomes. Small 2014;**10**(8):1575-84 doi: 10.1002/smll.201303433[published Online First: Epub Date]|.

31. Abrishami M, Zarei-Ghanavati S, Soroush D, Rouhbakhsh M, Jaafari MR, Malaekeh-Nikouei B. Preparation, characterization, and in vivo evaluation of nanoliposomes-encapsulated bevacizumab (avastin) for intravitreal administration. Retina 2009;**29**(5):699-703 doi: 10.1097/IAE.0b013e3181a2f42a[published Online First: Epub Date]|.

32. Altamirano-Vallejo JC, Navarro-Partida J, Gonzalez-De la Rosa A, et al. Characterization and Pharmacokinetics of Triamcinolone Acetonide-Loaded Liposomes Topical Formulations for Vitreoretinal Drug Delivery. J Ocul Pharmacol Ther 2018;**34**(5):416-25 doi: 10.1089/jop.2017.0099[published Online First: Epub Date]|.

33. Araujo J, Garcia ML, Mallandrich M, Souto EB, Calpena AC. Release profile and transscleral permeation of triamcinolone acetonide loaded nanostructured lipid carriers (TA-NLC): in vitro and ex vivo studies. Nanomedicine 2012;**8**(6):1034-41 doi: 10.1016/j.nano.2011.10.015[published Online First: Epub Date]|.

34. Sherje AP, Jadhav M, Dravyakar BR, Kadam D. Dendrimers: A versatile nanocarrier for drug delivery and targeting. Int J Pharm 2018;**548**(1):707-20 doi: 10.1016/j.ijpharm.2018.07.030[published Online First: Epub Date]|.

35. Marano RJ, Toth I, Wimmer N, Brankov M, Rakoczy PE. Dendrimer delivery of an anti-VEGF oligonucleotide into the eye: a long-term study into inhibition of laser-induced CNV, distribution, uptake and toxicity. Gene Ther 2005;**12**(21):1544-50 doi: 10.1038/sj.gt.3302579[published Online First: Epub Date]|.

36. Yavuz B, Bozdag Pehlivan S, Sumer Bolu B, Nomak Sanyal R, Vural I, Unlu N. Dexamethasone - PAMAM dendrimer conjugates for retinal delivery: preparation, characterization and in vivo evaluation. J Pharm Pharmacol 2016;**68**(8):1010-20 doi: 10.1111/jphp.12587[published Online First: Epub Date]|.

37. Mandal A, Bisht R, Rupenthal ID, Mitra AK. Polymeric micelles for ocular drug delivery: From structural frameworks to recent preclinical studies. J Control Release 2017;**248**:96-116 doi: 10.1016/j.jconrel.2017.01.012[published Online First: Epub Date]|.

38. Vaishya RD, Gokulgandhi M, Patel S, Minocha M, Mitra AK. Novel dexamethasone-loaded nanomicelles for the intermediate and posterior segment uveitis. AAPS PharmSciTech 2014;**15**(5):1238-51 doi: 10.1208/s12249-014-0100-4[published Online First: Epub Date]|.

39. Famili A, Kahook MY, Park D. A combined micelle and poly(serinol hexamethylene urea)-co-poly(N-isopropylacrylamide) reverse thermal gel as an injectable ocular drug delivery system. Macromol Biosci 2014;**14**(12):1719-29 doi: 10.1002/mabi.201400250[published Online First: Epub Date]|.

40. 2018 hcgcsNaoO.

41. Humayun M, Santos A, Altamirano JC, et al. Implantable MicroPump for Drug Delivery in Patients with Diabetic Macular Edema. Transl Vis Sci Technol 2014;**3**(6):5 doi: 10.1167/tvst.3.6.5[published Online First: Epub Date]|.

42. Guidotti G, Brambilla L, Rossi D. Cell-Penetrating Peptides: From Basic Research to Clinics. Trends Pharmacol Sci 2017;**38**(4):406-24 doi: 10.1016/j.tips.2017.01.003[published Online First: Epub Date]|.

43. Reissmann S. Cell penetration: scope and limitations by the application of cell-penetrating peptides. J Pept Sci 2014;**20**(10):760-84 doi: 10.1002/psc.2672[published Online First: Epub Date]|.

44. de Cogan F, Hill LJ, Lynch A, et al. Topical Delivery of Anti-VEGF Drugs to the Ocular Posterior Segment Using Cell-Penetrating Peptides. Invest Ophthalmol Vis Sci 2017;**58**(5):2578-90 doi: 10.1167/iovs.16-20072[published Online First: Epub Date]|.

45. Zhang X, Li Y, Cheng Y, et al. Tat PTD-endostatin: A novel anti-angiogenesis protein with ocular barrier permeability via eye-drops. Biochim Biophys Acta 2015;**1850**(6):1140-9 doi: 10.1016/j.bbagen.2015.01.019[published Online First: Epub Date]|.

46. Chen C, Liu K, Xu Y, et al. Anti-angiogenesis through noninvasive to minimally invasive intraocular delivery of the peptide CC12 identified by in vivo-directed evolution. Biomaterials 2017;**112**:218-33 doi: 10.1016/j.biomaterials.2016.09.022[published Online First: Epub Date]|.

47. Suda K, Murakami T, Gotoh N, et al. High-density lipoprotein mutant eye drops for the treatment of posterior eye diseases. J Control Release 2017;**266**:301-09 doi: 10.1016/j.jconrel.2017.09.036[published Online First: Epub Date]|.

48. Pescina S, Ostacolo C, Gomez-Monterrey IM, et al. Cell penetrating peptides in ocular drug delivery: State of the art. J Control Release 2018;**284**:84-102 doi: 10.1016/j.jconrel.2018.06.023[published Online First: Epub Date]|.

4

Evaluation of subconjunctival liposomal steroids for the treatment of experimental uveitis

Chee Wai Wong[1,2†]

Bertrand Czarny[3,4,5 †]

Josbert M. Metselaar[6]

Candice Ho[2]

Ng Si Rui[1,2]

Amutha Barathi Veluchamy[2]

Gert Storm[3,6*]

Tina T. Wong[1,2*]

1. Singapore National Eye Centre (SNEC), 11 Third Hospital Avenue, Singapore 168751
2. Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore 168751
3. Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands.
4. School of Materials Science and Engineering (MSE), Nanyang Technological University, 11 Faculty Avenue, Singapore 639977
5. Lee Kong Chian school of medicine (LKCmedicine), Nanyang Technological University, 11 Mandalay Road, Singapore 308232
6. Department of Experimental Molecular Imaging, University Clinic and Helmholtz Institute for Biomedical Engineering, RWTH Aachen University, Aachen 52074, Germany

Sci Rep. 2018 Apr 26;8(1):6604.

Abstract

Non-infectious anterior uveitis (AU) is a potentially sight threatening inflammatory condition. The current gold standard for treatment is topical steroids, but low ocular bioavailability and compliance issues with the intensive dosing regimen limit the efficacy of this treatment. Liposomes as a drug delivery system may help to overcome these problems. We studied the efficacy of a PEG-liposomal formulation of liposomal steroids, administered as a single subconjunctival dose, in the treatment of experimental uveitis in rabbit eyes. Rabbits that received subconjunctival liposomal triamcinolone acetonide phosphate (LTAP) or liposomal prednisolone phosphate (LPP) had significantly lower mean inflammatory scores than untreated controls on Day 4 after induction of uveitis (LPP vs controls, $p=0.049$) and 8 (LPP vs controls, $p=0.007$; LTAP vs controls, $p= 0.019$), and lower scores than rabbits given topical PredForte1% 4 times a day on Day 8 ($p=0.03$). After antigen rechallenge, the subconjunctival liposomal steroid groups continued to have greater suppression of inflammation than untreated controls on Day 11 ($p=0.02$). Localization of liposomes in inflamed ocular tissue was confirmed by histology and immunostaining, and persisted in the eye for at least one month. Our study demonstrates that a single subconjunctival injection of liposomal steroids induces effective and sustained anti-inflammatory action.

Background

Non-infectious anterior uveitis (AU) represents a group of immune-related, sight-threatening inflammatory conditions that account for 60% of all cases of uveitis seen in eye centres.[1–5] Sight threatening eye complications can occur upon prolonged uncontrolled inflammation, including cataract, glaucoma, and swelling of the central retina. These complications lead to blindness in up to 25% of patients.[7] Corticosteroids are the first choice treatment for anterior uveitis, and the current gold standard treatment is topical eyedrops therapy.[8] However, several limitations exist for topical eyedrop administration[9]: 1. Bioavailability is poor due to limited capacity of the conjunctival sac (25µl), short precorneal drug residence time and drainage of drug via the nasolacrimal duct. It has been estimated that the ocular absorption of topically applied drugs is less than 5%[10]; 2. Steroid eye drops are suspensions, which can cause blurring of vision and ocular irritation[11]; 3. Intensive treatment is required, resulting in a challenge to comply with the treatment regimen. These factors combine to limit the efficacy of topical eyedrop treatment, resulting in persistent inflammation and sight threatening complications related to chronic inflammation. In addition, the untargeted delivery of steroids to uninflamed ocular tissue can result in steroid related side effects such as cataract[11] and glaucoma.[8]

To avoid the problems of poor bioavailability as well as the side effects, various drug delivery systems have been studied for treating ophthalmic diseases, including polymer- and lipid-based nanomaterials.[9,12–14] The most studied nanocarriers in ophthalmic disease are liposomes, which have the advantages of being biocompatible and biodegradable.[14] Some liposomal formulations are already used in clinical trials for eye diseases.[15,16] Different routes of delivery and formulations have been developed to optimize the delivery of liposomal drugs into the anterior or posterior segment of the eye by altering the surface charge or lipid composition.[15,17] The use of liposomal formulations of vasoactive intestinal peptide[18,19], dichloromethylene-diphosphonate[20–23] and FK506[24] for the treatment of experimental

uveitis have been previously reported. Our study is the first to assess the effectiveness of liposomes to deliver a single subconjunctival dose of a well-established treatment (steroid), in comparison to a single injection of unencapsulated steroid and to the current gold standard of intensive topical steroid eyedrops.

In this study, we employed pegylated liposomal formulations of water-soluble corticosteroid derivatives, notably prednisolone phosphate and triamcinolone acetonide phosphate, both active ingredients know to be effective in free form for the treatment of AU in humans. PEGylation of liposomes enhances their bioavailability by increasing solubility, decreasing enzymatic degradation and reducing clearance.[25] Pegylated liposomal formulations of water-soluble corticosteroids have already shown promising results in human trials to treat systemic inflammatory diseases such as rheumatoid arthritis (Phase I/II) ~~a~~nd ulcerative colitis (Phase IIa).[26,27] Interestingly these liposomes have been shown to be efficiently and specifically taken up by macrophages in inflamed tissue.[22,28,29] When injected in the subconjunctival space, we postulate that, besides creating a local depot providing sustained release of the drug, the liposomes could also enhance uptake of the drug by local inflammatory target cells in AU. This is a therapeutic efficacy study with a GMP-liposomal corticosteroid formulation that has shown to be active in a variety of models of chronic inflammation after IV administration, by virtue of targeting to the inflamed target sites.[26,27,29-31] In this study, we compared the efficacy of subconjunctival liposomal prednisolone phosphate (LPP) and liposomal triamcinolone acetonide phosphate (LTAP) with topical prednisolone acetate 1% eyedrops and subconjunctival free prednisolone phosphate for the treatment of AU in a rabbit model of experimental uveitis.

Materials and Methods

Animals

Approval was obtained from the SingHealth Institute Animal Care and Use Committee (IACUC Singhealth Approval Number 2016/SHS/1184) and all procedures were performed in accordance with

the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. 26 Adult New Zealand White rabbits, weighing 2-2.5kg were used in this study. All rabbits were examined with a slit lamp and only rabbits with no ocular pathology were included in the study. Rabbits were randomized into one of the following arms: Subconjunctival liposomal prednisolone phosphate, subconjunctival liposomal triamcinolone acetonide, subconjunctival free prednisolone phosphate, topical prednisolone acetate 1% and no treatment.

Liposomal steroid preparation

Liposomes were prepared as previously described.[30] In brief, dipalmitoyl phosphatidyl choline (DPPC), cholesterol, and PEG2000 distearoyl phosphatidylethanolamine (PEG-DSPE) were added in a 62%, 33%, and 5% molar ratio. Steroids were dissolved in water for injection while the lipids were dissolved in absolute ethanol at 65 °C. The alcoholic lipid solution was injected in the aqueous steroid solution and mixed under heating to 65 °C, forming a multilamellar vesicle dispersion. This dispersion was downsized to the desired particle size of approximately 100 nm in diameter by repeated homogenization cycles using an Avestin C55 high-shear homogenizer (Avestin, Mannheim, Germany). Unencapsulated steroids were removed by ultrafiltration using membranes with a molecular weight cut off of 30 kDa and replaced with clean dispersion buffer. Finally, the liposomal dispersion was sterile filtered, collected in vials and stored between 2 and 8 °C. Cyanine 5.5 and Fluorescein isothiocyanate (FITC) liposomes were prepared identically with the addition of 0.2% of DSPE-CY5.5 or FITC as described by Lobatto et al.[31] The characteristics (Table 1) and drug release profile of this formulation in aqueous medium and plasma has previously been published. In such media, they show good drug retention properties, which is essential to ensure transport and delivery of the liposomal encapsulated drug at the target cells (e.g. macrophages) in the inflamed site.[26,27,29] The formulation studied here is the same as the formulation developed and evaluated by Lobatto et al. With this formulation, neither in vitro (buffer, 37 °C) nor in vivo (in the blood circulation) release of encapsulated drug from the liposomes was observed.[31] However, despite the complete stability of the liposomes in vitro and in the circulation, low levels of free drug were detected in plasma, which are due to liposome clearance from blood and subsequent drug release by liver and spleen macrophages back to the circulation.[26]

Table 1: Characteristics of liposomes

	Empty liposomes (C)	Prednisolone liposomes (LPP)	Triamcinolone liposomes (LTAP)	CY5.5 liposomes	FITC liposomes
Size (nm)	120 ± 5	110 ± 6	110 ± 2	114 ± 2	132 ± 2
PDI	0.014	0.040	0.100	0.060	0.010
Zeta potential (meV)	-0.6	+4.3	+5.5	+0.5	-0.3
Drug concentration	-	5mg/ml	5mg/ml	-	-
Encapsulation Efficiency (EE%)	-	10%	10%	-	-

Preimmunization

A subcutaneous injection of *Mycobacterium tuberculosis* H37Ra antigen (10mg; Difco, Detroit, MI) suspended in mineral oil (500µL) was given as preimmunization.[32] One week later, a second injection of the same amount of subcutaneous antigen was given at a separate site. Successful preimmunization was confirmed after one week by the presence of a visible skin nodule at the injection site.

Induction of experimental uveitis

Experimental uveitis was induced by unilateral intravitreal injection on Day 0 in preimmunized rabbits (7 days after the second preimmunization). The rabbits were anesthetized with intraperitoneal injections of ketamine hydrochloride (35- 50mg/kg) and Xylazil (5- 10mg/kg). Following topical anaesthesia with Amethocaine 1%, the right eye of each rabbit was disinfected with 5% povidone iodine. An intravitreal injection of *Mycobacterium tuberculosis* H37Ra antigen suspended in sterile saline (50µg; 1µg/µL) using a Hamilton syringe with a 31-gauge needle was given through the superotemporal sclera, 1.5mm from the limbus. One drop of Tobramycin was instilled at the end of the procedure. To simulate a recurrence of uveitis, we induced experimental uveitis again on Day 8, following the procedure as

described above. The eyes were clinically monitored for 30 days and graded for ocular inflammation by 2 masked investigators.

Kinetics profile and localization of liposomes

Fluorescent-labelled liposomes were injected subconjunctivally in 4 rabbits to investigate their ocular distribution. Two rabbits received a subconjunctival injection of liposomes labelled with Cyanine 5.5 and were sacrificed 24h later. Eyes were frozen and sliced for immunostaining and confocal imaging (Nikon center Singapore). Two rabbits were injected with liposomes labelled with FITC to observe the kinetics at the subconjunctival injection site, the cornea and the aqueous humor with a fluorotron imaging. The Fluorotron Master (Fluorophotometry equipment) is approved for human use and this version only differs slightly from the human version in its external features that make it appropriate for positioning to animal eyes. Briefly, 200 µl of FITC labelled liposome solution were injected subconjunctivally into both eyes. Concentration measurements were undertaken with the Fluorotron at baseline, 15 min, 60 min, 48 hours and weeks 1,2,3 and 4 post injection for the cornea and aqueous humor sites. Two extra time points (4h and 24 hours) were added for the subconjunctival injection site.

Intervention

Rabbits were randomized into 5 groups, 3 days after uveitis induction: a single dose of 0.1ml subconjunctival LTAP (4mg/ml) (n=6), a single dose of 0.1ml subconjunctival LPP (4mg/ml) (n=5), a single dose of 0.1ml of subconjunctival prednisolone phosphate (FPP) (4mg/ml), topical Predforte1% Q3H for 2 weeks (ED) (n=5) or controls (C) (n=5). Prior to injection, rabbits were anesthetized with intraperitoneal injections of ketamine hydrochloride (35- 50mg/kg) and Xylazil (5- 10mg/kg). Following topical anaesthesia with Amethocaine 1%, the right eye of each rabbit was disinfected with 5% povidone iodine. A Hamilton syringe with a 31-gauge needle was used to deliver subconjunctival injections. Topical Tobramycin was administered 4 times a day for 5 days after the subconjunctival injection.

Ocular examination

Ocular examination was performed by 2 masked independent investigators (CW, SR). Slit-lamp biomicroscopy, measurement of intraocular pressure with the Tonopen, photography of the anterior segment and dilated fundal examination with binocular indirect ophthalmoscopy using a 20D lens were performed prior to uveitis induction and at 8 defined time points thereafter (Days 0, 1, 3, 4, 8, 9, 11, 16, 24 and 31). Severity of uveitis was scored by evaluating anterior chamber cells/flares, vitreous haze, and iris vessels. These clinical scoring systems had been described in previous literature.[33,34] The combined anterior segment inflammation score was defined as the sum of the scores for iris vessels, anterior chamber cells and anterior chamber flare. The presence of cataract was determined on slit lamp biomicroscopy on day 31 and graded based on the LOCS scale.

Enucleation, euthanasia & pathology procedures

All rabbits were euthanized at the end of the study period of 30 days. Euthanasia was carried out with intraperitoneal pentobarbitone (60-150mg/kg) followed by enucleation of the operated eyes.

Histopathology and immunohistochemistry

Eye were embedded in paraffin or directly frozen (eyes injected with Cy5.5 labelled liposomes). For paraffin embedding, the enucleated rabbit eye was fixed in 10% neutral buffered formalin solution (Leica Surgipath, Leica Biosystems Richmond, Inc.) for 24 hours. The whole rabbit eye was then dissected prior to dehydration in increasing concentrations of ethanol, clearance in xylene, and embedding in paraffin (Leica-Surgipath, Leica Biosystems Richmond, Inc.) Five-micron sections were cut with a rotary microtome (RM2255, Leica Biosystems Nussloch GmbH, Germany) and collected on POLYSINE^{TM} microscope glass slides (Gerhard Menzel, Thermo Fisher Scientific, Newington, CT). The sections were dried in an oven of 37^{o}C for at least 24 hours. To prepare the sections for histopathological and immunohistochemical examination, the sections were heated on a 60^{o}C heat plate, deparaffinized in xylene and rehydrated in decreasing concentration of ethanol.

For directly frozen eyes, the whole rabbit eye was embedded in Optimal Cutting Temperature (OCT) compound at -20 ° C for 1 hour. Six-micron sections were cut with a cryostat (HM550, Thermo Fisher Scientific Microm International GmbH, Germany) and collected on POLYSINE^{TM} microscope glass slides (Gerhard Menzel, Thermo Fisher Scientific, Newington, CT). Sections were air dried at room temperature (RT) for 1 hour.

A standard procedure for Hematoxylin and Eosin (H&E) was performed. A light microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides and images were captured. In parallel, immunofluorescence staining was performed. For paraffin, heat-induced antigen retrieval was performed by incubating sections in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) for 20 minutes at 95-100ºC. The sections were then cooled down in sodium citrate buffer for 20 minutes in RT and washed three times for 5 minutes each with 1X phosphate buffered solution (PBS). For frozen samples, the sections were fixed in 4% paraformaldehyde (PFA) in 1X PBS for 10 minutes and washed three times for 5 minutes each with 1X PBS.

Non-specific sites were blocked with blocking solution of 5% bovine serum albumin (BSA) in 0.1% Triton X-100 and 1XPBS for 1 hour at room temperature in a humidified chamber. The slides were then rinsed briefly with 1X PBS. A specific primary antibody as shown in supplementary table S1 was applied and incubated overnight at 4ºC in a humidified chamber prepared in blocking solution. After washing twice with 1X PBS and once with 1X PBS with 0.1% tween for 10 minutes each, Alexa Fluro® 488/594 – conjugated fluorescein-labelled goat anti-rabbit IgG secondary antibody (Invitrogen-Molecular Probes, Eugene, OR) was applied at a concentration of 1:1000 in blocking solution and incubated for 90 minutes at RT. The slides were then washed twice with 1X PBS and once with 1X PBS with 0.1% tween for 5 minutes each, the slides were mounted on the slides with Prolong Diamond Anti-fade DAPI5 Mounting Media (Invitrogen- Molecular Probes, Eugene, OR) to visualize cell nuclei. For negative controls, primary antibody was omitted.

A confocal microscope system (Nikon A1R+si Confocal Microscope) was used to capture high-resolution images. Experiments were repeated in duplicates for four antibodies.

Statistical Analysis

The main outcome measure was the combined clinical scores, defined as the sum of the following scores: 1) iris vessels, 2) anterior chamber cells and 3) anterior chamber flare. Secondary outcome measures were mean intraocular pressure and proportion of eyes with cataract. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 20 program. Ordinal variables were described with means and analyzed using Mann Whitney U test for independent samples. Proportions were analyzed with the chi square test All p-values are 2 sided with appropriate significance of $p<0.05$.

Results

Inflammatory scores

Table 2 shows the mean combined anterior segment inflammatory scores. One day after subconjunctival injection, the combined anterior segment inflammatory score was significantly lower in the liposomal PP group than in the controls (5.4±1.5 vs 8.4±1.7, p=0.049), and was also significantly lower than in the eyedrops group (p=0.033) This difference persisted for 5 days after initial intervention, with both liposomal groups (2.6±2.1, p=0.019 and 3.3±2.5, p=0.024 in the liposomal PP and liposomal TA groups respectively) demonstrating significantly lower combined anterior segment inflammatory scores than controls (7.2±2.2). Liposomal PP achieved greater attenuation of rebound inflammation than controls on day 11, 3 days after a rechallenge with intravitreal TB antigen (4.7±2.6 vs 8.5±1.3, p=0.041). In comparison, while subconjunctival free PP was able to suppress inflammation significantly on day 8 (3.2±0.4), rebound inflammation was observed on day 11 (7.0±2.3). A single dose of subconjunctival liposomal PP or TA delivered sustained anti-inflammatory for 2 weeks post treatment, similar to daily Pred forte eyedrops instilled 4 times a day for 2 weeks (5.0±2.8 and 5.0±1.0 for liposomal PP and TA

respectively, vs 4.6±1.3 for eyedrops, $p>0.05$). Slit lamp and fundus photographs of all treatment groups are shown in Figure 1. The control eye showed greater iris congestion, anterior chamber cells and flare and vitreous haze compared to the eye treated with liposomal PP, 1 week after initiation of treatment (Day 11). Figure 2 shows the mean change in combined anterior segment inflammatory scores relative to the maximum inflammation on day 3. Decreases in combined anterior segment inflammatory scores relative to the score on day 3 were greatest in both liposomal groups one day and 5 days after treatment. In addition, there was greatest attenuation of rebound inflammation after antigen re-challenge again in both liposomal groups. On day 16, both liposomal groups achieved a similar decrease in mean inflammatory scores compared to the eyedrop group.

Table 2: Mean combined anterior segment inflammatory scores.

Day	**Combined anterior segment inflammatory score**					
	Liposomal PP (n=5)	Liposomal TA (n=6)	Free PP (n=5)	Pred Forte 1% eyedrops (n=5)	Controls (n=5)	†P
0	**1st intravitreal induction**					
1	9.4±0.5	9.7±0.5	8.6±0.5	9.0±1.0	9.6±0.5	0.080
3	9.4±0.5	9.7±0.5	8.0±0.7	9.0±1.4	9.0±1.4	0.350
3	**Intervention**					
4	5.4±1.5*	6.5±1.9	6.0±0.7	8.0±1.4*	8.4±1.7*	0.020

8	2.6±2.1**	3.3±2.5**	3.2±0.4**	6.0±0.7	7.2±2.2**	0.002
8	**2nd intravitreal induction**					
9	5.8±2.7	7.0±2.4	8.0±1.2	8.8±1.3	8.5±2.4	0.130
11	4.7±2.6***	5.5±2.3	7.0±2.3	6.4±0.9	8.5±1.3***	0.041
16	5.0±2.8	5.0±1.0	5.4±1.3	4.6±1.3	7.6±1.9	0.080
24	1.4±1.5	2.2±1.7	2.8±0.4	1.2±1.6	4.0±2.2	0.080
31	0.8±1.8	2.0±2.5	2.2±1.3	1.8±1.9	3.2±1.8	0.440

†p values from one-way ANOVA, comparing mean combined anterior segment inflammatory scores between groups.

*Pairwise comparison between liposomal PP with controls, p=0.049 and with eyedrops, p=0.033 ** Pairwise comparison between liposomal PP with controls, p=0.007, pairwise comparison between liposomal TA with controls, p=0.019 and pairwise comparison between free PP with controls (p=0.024)

*** Pairwise comparison between liposomal PP with controls, p=0.041

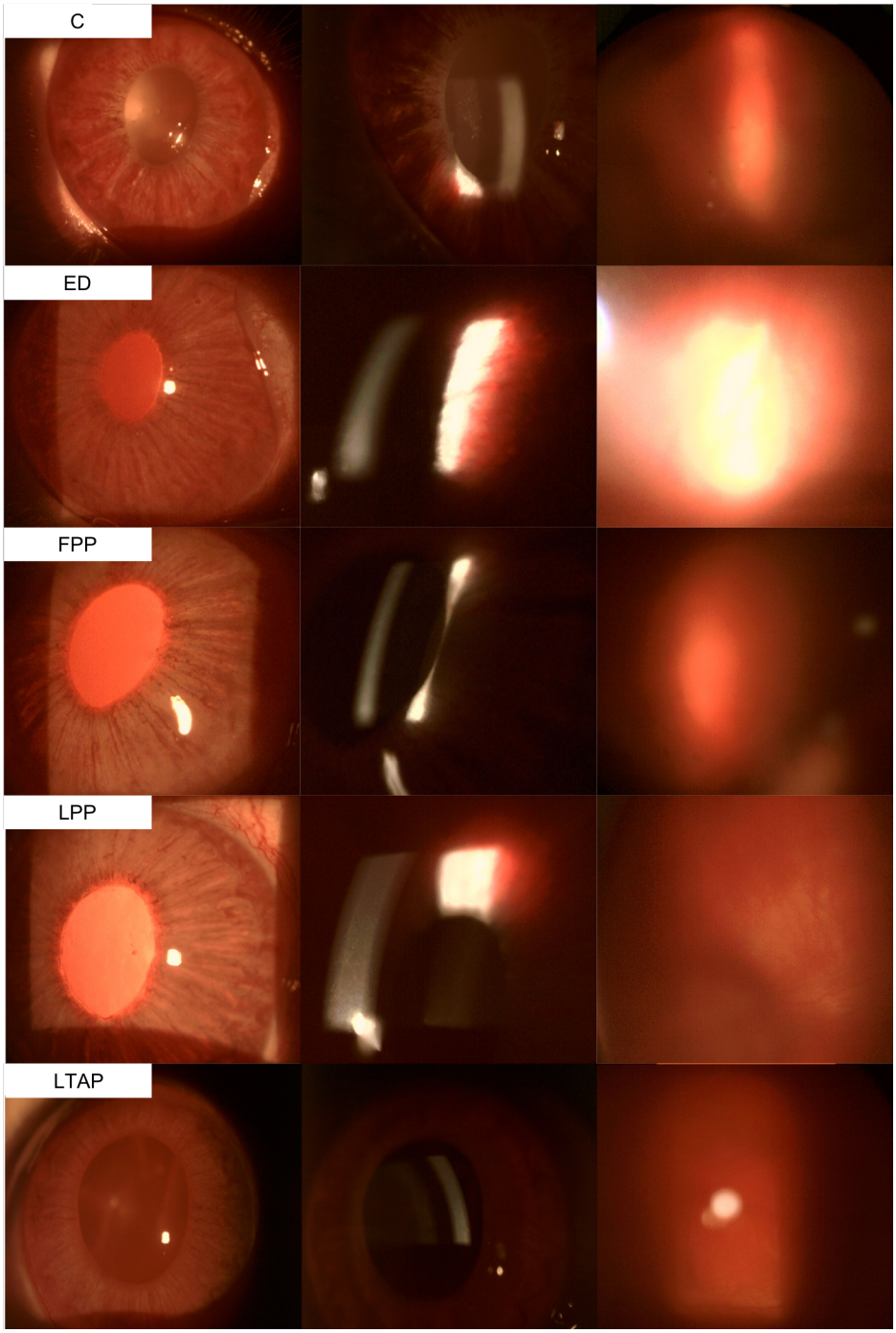

Figure 1: Slit lamp (left and middle rows) and fundus photos (right row) on Day 11. Control eye (top row) had greater iris congestion (top left), anterior chamber cells and flare (top mid) than the eye treated with liposomal PP. Vitreous haze was also worse in the control eye (top right vs 4th row right). Abbreviations: control PBS (C), free prednisolone phosphate (FPP), liposomal prednisolone phosphate (LPP), liposomal triamcinolone phosphate (LTAP), Eye drops treatment (ED).

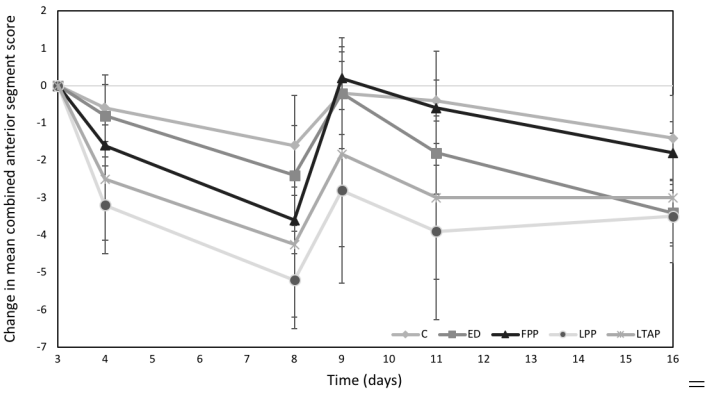

Figure 2: Mean change in combined anterior segment inflammatory scores**,** (normalization to maximum inflammation on day 3). Treatment started on day 3 with one subconjunctival injection of liposomal prednisolone phosphate (LPP), control PBS (C), free prednisolone phosphate (FPP) or liposomal triamcinolone phosphate (LTAP). Eye drops treatment (ED) started on day 3 with Q3H/4 drops per day until day 16. Recurrence of inflammation was simulated on Day 8 with a repeat challenge of TB antigen.

Cataract formation

Overall, posterior subcapsular cataracts developed in 11 rabbits. No other subtype of cataract was observed. There was no significant difference in the rate of cataract formation between treatment groups (p=0.185) but there was a trend towards higher rates in controls, eyedrops and subconjunctival free prednisolone phosphate groups (Figure 3). No cataracts were seen in fellow eyes administered with prednisolone acetate 1% eyedrops.

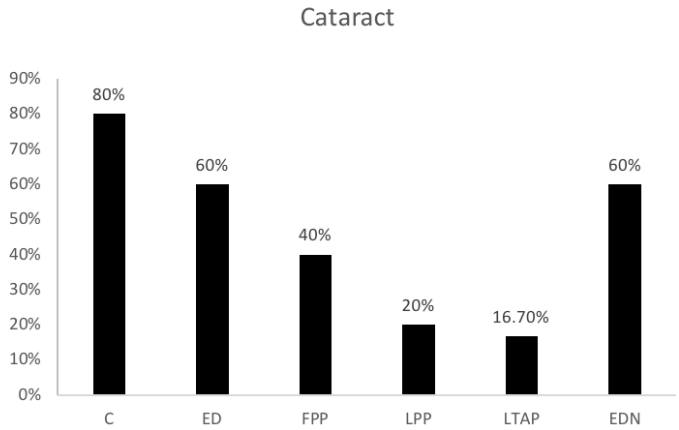

Figure 3: Cataract formation by treatment group. Abbreviations: control PBS (C), free prednisolone phosphate (FPP), liposomal prednisolone phosphate (LPP), liposomal triamcinolone phosphate (LTAP), Eye drops treatment (ED), Eye drops treatment in non-inflamed eye (EDN). Treatment started on day 3 with one subconjunctival injection or eye drops 4 times a day until day 16.

Intraocular pressure

There were no significant differences in IOP between the treatment groups (Figure 4) at any time point. A non-significant spike in IOP was observed on day 9, a day after antigen-rechallenge, in all groups except the control group and in the non-inflamed eyes, but IOP remained within normal limits in all eyes. Importantly, none of the rabbits experienced an IOP>21 at any point during the experiment.

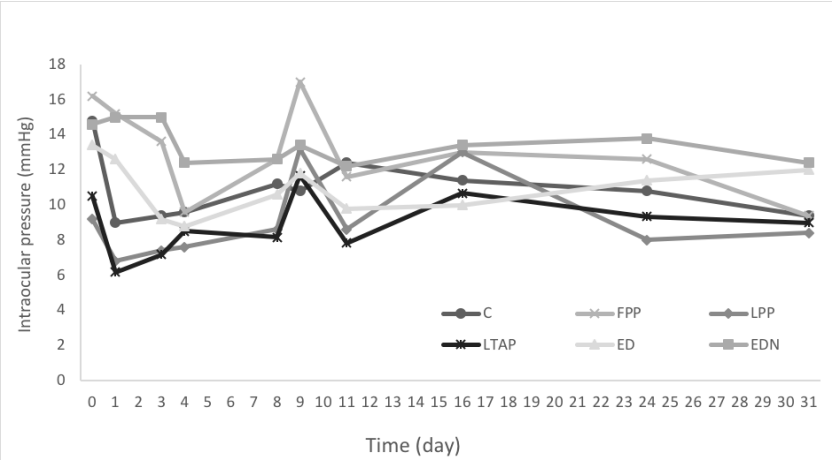

Figure 4: Intra Ocular Pressure (IOP) in each treatment group over time. Control PBS (C), free prednisolone phosphate (FPP), liposomal prednisolone phosphate (LPP), liposomal triamcinolone phosphate (LTAP), Eye drops treatment (ED), Eye drops treatment in non-inflamed eye (EDL). Treatment started on day 3 with a single subconjunctival injection or eye drops 4 times a day until day 16. Recurrence of inflammation was simulated on Day 8 with a repeat challenge of TB antigen.

Histology and immunohistochemical staining

The H&E staining showed normal tissue structure in all groups. However, more cellular infiltration (dark purple) was observed in the control group. With immunohistochemical staining, we confirmed ciliary body inflammation in the control group with presence of leucocytes (CD45, figure 5B1) and T lymphocytes (CD4). The number of inflammatory cells between groups correlated with the observed inflammatory score (Figure 5A, B&C): less inflammatory cells were seen in the LPP and LTAP groups compared with ED, free PP or controls at 30 days post uveitis induction (Figure 5C).

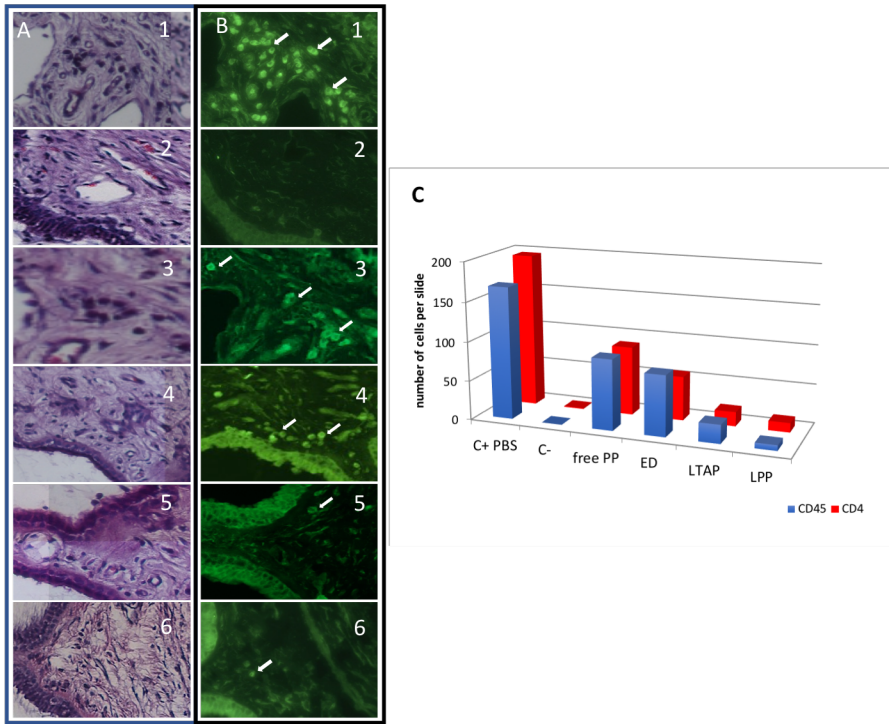

Figure 5: inflammatory response to treatment, day 30. **(**A) HE staining of each treatment, (B) immunostaining with anti-CD45. PBS (1), healthy eye (2), free prednisolone, FPP (3), prednisolone eye drops, ED (4), liposomal triamcinolone phosphate, LTAP (5), liposomal prednisolone phosphate, LPP (6). Inflammatory cells in treated eye (white arrow). **(**C) Mean number of CD45 and CD4 cells present in the ciliary body per slide after treatment on day 30 for each treatment. Control PBS (C+PBS), normal fellow eye (C-), eyedrops (ED), free prednisolone phosphate (free PP), liposomal triamcinolone acetonide phosphate (LTAP), liposomal prednisolone phosphate (LPP).

Localisation of liposomes in inflamed areas

24 hours after injection of CY5.5-liposomes, fluorescence was detected in the ciliary body as well as the subconjunctival injection area. After immunostaining, fluorescence was detected within macrophages (figure 6).

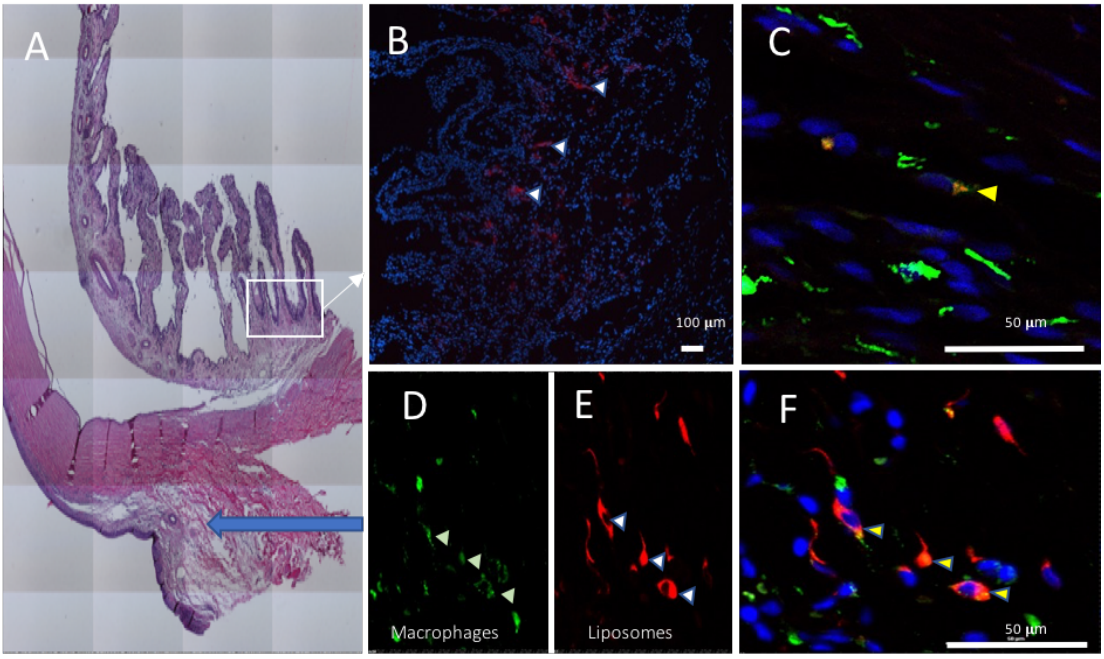

Figure 6: Localization of liposomes in PBS group (Control) on day 4, 24 hours after liposomes CY5.5 injection. (A) HE staining on the anterior segment, in paraffin. Blue arrow represents the subconjunctival injection site. White square represents the ciliary body. (B, C) Confocal imaging focused on the ciliary body (white square), frozen tissue. (D, E, F) Confocal imaging focused on subconjunctival injection site (blue arrow) (D) staining of macrophages (green), (E) liposomes (red), (F) overlay. Liposomes are represented in red (white arrows), nucleus in blue (DAPI), macrophages in green (Alexa 594), yellow arrow shows the co-localisation of macrophages and liposomes.

Liposome kinetics

FITC labelled liposomes, after injection in the subconjunctival space, showed a fast elimination during the first day followed by slow elimination persisting over the entire duration of the experiment from the subconjunctival area. A lower quantity of FITC-labelled liposomes was detected in the

cornea and this was maintained over 4 weeks. In the aqueous humor, an equivalent quantity was measured and maintained over time, but FITC was detected only after 24 hours post subconjunctival injection (figure 7).

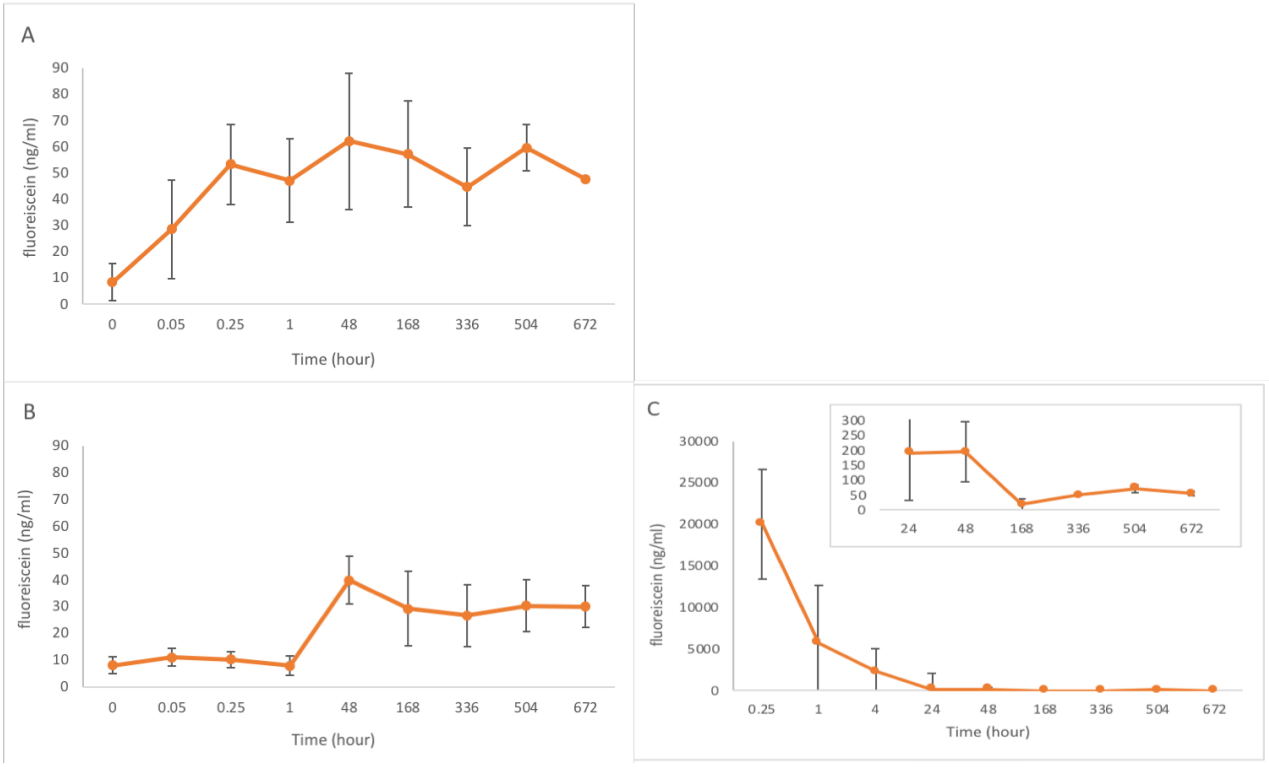

Figure 7: Kinetics of FITC- labelled liposomes after subconjunctival injection. Mean fluorescein concentration (ng/ml) measured over time (hours) with fluorophotometry. (A) in the cornea, (B) in aqueous, (c) in the subconjunctival space.

Discussion

In this study, we demonstrated that a single dose of liposomal steroid, injected subconjunctivally, was able to provide sustained anti-inflammatory action comparable to 2 weeks of eyedrop therapy with prednisolone acetate 1%. Importantly, we found that liposomal prednisolone phosphate was able to suppress the initial inflammation better than eyedrops ($p=0.033$). This is likely the result of a more rapid build-up of therapeutic levels within the eye via the subconjunctival route compared to topical administration. The subconjunctival route may also reduce ocular irritation associated with

benzalkonium chloride, a preservative commonly found in topical eyedrops. Further, we observed that liposomal steroid was able to sustain anti-inflammatory action and attenuate an antigen rechallenge, an effect that was not achieved by subconjunctival injection or topical application of free steroid. Subconjunctival injections can be given relatively easily and painlessly in the outpatient setting under topical anaesthesia. There is little risk of globe injury compared to peribulbar injections. Moreover, as there is no intraocular penetration, subconjunctival administration does not entail the risk of endophthalmitis that is associated with intravitreal or intracameral injections.

A myriad of drug delivery approaches has previously been studied for the delivery of corticosteroids to treat anterior uveitis. These include cubosomes[35], micellar systems, implant[12], a variety of nanoparticles, microemulsions, and iontophoresis. At present, only iontophoresis has been evaluated in phase III trials for the treatment of anterior uveitis, demonstrating non-inferiority when compared to intensive topical eyedrop therapy.

Liposomal steroids, whether local or systemic, have not been previously assessed for the treatment of anterior uveitis. Liposomes are one of the more successful drug delivery platforms for ocular diseases that have made it to human clinical trials, including their application to treat dry eyes[36], allergic rhinoconjunctivitis[37] and cytomegalovirus infection of the retina[38]. In earlier studies, Pouvreau et al[22] and Broekhuyse et al[21] have observed a significant anti-inflammatory effect on experimental uveitis after depletion of macrophages with dichloromethylene diphosphonate (Cl2MDP)-containing liposomes. These results suggest that liposomes may have the advantage of preferential uptake by macrophages, the major cell type involved in anterior uveitis, as demonstrated in our study with the observation of co-localisation of liposomes and macrophages.

An added advantage of the subconjunctival administration route may be the avoidance of ocular side effects. While steroids are crucial for the adequate suppression of inflammation-related, they can cause sight-threatening complications such as raised intraocular pressure and cataracts. Previous studies have shown that 30% of all steroid-treated eyes may experience elevation of intraocular

pressure after prolonged topical steroid treatment. This side effect is related to a direct effect of steroids on the extracellular matrix proteins in the trabecular meshwork and the inhibition of phagocytosis by trabecular meshwork cells, both of which cause a reduction in aqueous outflow.[39,40] In our study, none of the eyes treated with liposomal steroids developed raised intraocular pressure throughout the duration of the study. Preferential uptake of liposomes by macrophages may reduce the total dose required for sufficient efficacy. In addition, encapsulation of steroids within liposomes may ameliorate some of the deleterious side effects when the trabecular meshwork is exposed to steroids. With regards to cataract formation, most eyes developed some degree of posterior subcapsular cataract in this study as a result of the inflammatory process, with fewer eyes experiencing cataract formation in the liposomal steroid groups. This can be attributed to the better control of inflammation in these eyes. None of the fellow eyes treated with liposomal steroid developed cataracts. Again, this may be attributed to the encapsulation of the steroid thus avoiding this adverse effect, but it may also result from the overall lowering of the dose of steroid via the single subconjunctival injection.

The tear film, cornea and anterior chamber and capillaries of the iris collectively form the ocular barriers to eye drop drug delivery for the treatment of anterior uveitis.[41,42] With subconjunctival injection, drug entry can potentially bypass the aforementioned barriers and, via crossing the sclera, reach the ciliary body, one of the two main target sites in anterior uveitis, the other being the iris. Indeed, liposomes have been reported to be able to cross the sclera and to reach the vitreous in intact form.[16,17] However, quantitatively, the extent to which the trans-scleral route permits drug delivery into the ciliary body is not known, and will most likely depend on the physicochemical properties of the drug delivery system in question. Drug binding to scleral melanin may form yet another barrier to drug delivery via the trans-scleral route. Regarding the mechanism behind the rapid and long lasting effect of both liposomal steroid preparations, we can only speculate about the various possible routes through which intact liposomes and steroid (still entrapped or released) reach the target inflammation areas (in the iris and the ciliary body) It is well established[26,27,30] that the type of liposomes used, PEGylated liposomes, can be taken up by inflammatory macrophages present in inflammatory lesions.

Our initial histological and immunochemical staining results confirm that the PEG-liposomes co-localise with macrophages at the injection site and in the ciliary body. Within these macrophages, the steroid-containing PEG-liposomes are degraded intracellularly. This intracellular degradation process occurring in the lysosomes liberates the entrapped steroid from the liposomal structures and released drug molecules are then able to diffuse throughout the cellular interior, and are possibly even released by the macrophages into the environment.[43,44] The released drug molecules act intracellularly to reduce the pro-inflammatory activity of these macrophages[26–28,31]. In addition to this mechanistic option, the administered PEG-liposomes could act as a depot slowly releasing its steroid content extracellularly. The exact sites of extracellular drug release are not known at present, but it has been previously described that, after subconjunctival injection of nanoparticles, released drugs are able to reach the ciliary body via the conjunctiva, tears and aqueous humour and also through the sclera and vitreous humor.[9]. Furthermore, the fluorotron results showed persistence of fluorescein (FITC-labelled liposomes) in the subconjunctival injection area over the entire duration of the experiment, indicating a sustained presence of liposomal nanoparticles at the injection site. This supports the notion that liposomes could act as a drug reservoir slowly releasing the drug at the injection site. We also detected a low but steadily maintained fluorescein quantity in the aqueous humour and in the cornea throughout the duration of the experiment.

There are limitations to our study. The study is not powered to study adverse effects. In addition, the follow up duration may be too short to identify the development of long term complications such as cataracts and raised IOP. However, a single subconjunctival injection of steroid is unlikely to induce cataracts, a complication seen with chronic topical steroid use or with intravitreal administration. Similarly, steroid induced IOP elevation is usually observed after a substantial period (several weeks to months) of topical steroid use[39], and is due to alterations in trabecular outflow resistance as discussed above[40]. This phenomenon may not be inducible in our animal model: a previous study failed to incite IOP elevation in rabbits with topical steroids applied 4 times a day for 1 month.[40] We propose that encapsulation of steroids in liposomes will reduce the total dose needed to achieve

efficacy, hence minimizing the exposure of the trabecular meshwork to the effects of steroids, and further reduce the likelihood of steroid-induced IOP elevation.

In conclusion, our study in a rabbit anterior uveitis model demonstrates that a single subconjunctival injection of liposomal steroids is capable of inducing effective and sustained anti-inflammatory action, and to attenuate the effects of a simulated recurrence of uveitis. Subconjunctival injections can be administered in the clinical setting safely with relative ease and without the need for sophisticated equipment. Our results suggest that a single subconjunctival injection of liposomal steroid represents an attractive option for the treatment of anterior uveitis and since these formulations are already under clinical investigation in other indications and administration routes, rapid translation of our preclinical results to a first-in-human trial may be possible.

References

1. Chang, J. H.-M. & Wakefield, D. Uveitis: a global perspective. *Ocul. Immunol. Inflamm.* **10,** 263–279 (2002).

2. Dandona, L., Dandona, R., John, R. K., McCarty, C. A. & Rao, G. N. Population based assessment of uveitis in an urban population in southern India. *Br. J. Ophthalmol.* **84,** 706–709 (2000).

3. Gritz, D. C. & Wong, I. G. Incidence and prevalence of uveitis in Northern California; the Northern California Epidemiology of Uveitis Study. *Ophthalmology* **111,** 491–500; discussion 500 (2004).

4. Päivönsalo-Hietanen, T., Tuominen, J., Vaahtoranta-Lehtonen, H. & Saari, K. M. Incidence and prevalence of different uveitis entities in Finland. *Acta Ophthalmol.* **75,** 76–81 (1997).

5. Tran, V. T., Auer, C., Guex-Crosier, Y., Pittet, N. & Herbort, C. P. Epidemiology of uveitis in Switzerland. *Ocul. Immunol. Inflamm.* **2,** 169–176 (1994).

6. Miserocchi, E., Fogliato, G., Modorati, G. & Bandello, F. Review on the worldwide epidemiology of uveitis. *Eur. J. Ophthalmol.* **23,** 705–717 (2013).

7. de Smet, M. D. *et al.* Understanding uveitis: the impact of research on visual outcomes. *Prog. Retin. Eye Res.* **30,** 452–470 (2011).

8. LeHoang, P. The gold standard of noninfectious uveitis: corticosteroids. *Dev. Ophthalmol.* **51,** 7–28 (2012).

9. Suresh, P. K. & Sah, A. K. Nanocarriers for ocular delivery for possible benefits in the treatment of anterior uveitis: focus on current paradigms and future directions. *Expert Opin. Drug Deliv.* **11,** 1747–1768 (2014).

10. Urtti, A. & Salminen, L. Minimizing systemic absorption of topically administered ophthalmic drugs. *Surv. Ophthalmol.* **37,** 435–456 (1993).

11. Athanasiadis, Y., Tsatsos, M., Sharma, A. & Hossain, P. Subconjunctival triamcinolone acetonide in the management of ocular inflammatory disease. *J. Ocul. Pharmacol. Ther.* **29,** 516–522 (2013).

12. Ang, M. *et al.* Evaluation of a prednisolone acetate-loaded subconjunctival implant for the treatment of recurrent uveitis in a rabbit model. *PLoS One* **9,** e97555 (2014).

13. Weng, Y. *et al.* Nanotechnology-based strategies for treatment of ocular disease. *Acta Pharm Sin B* **7,** 281–291 (2017).

14. Araújo, J., Nikolic, S., Egea, M. A., Souto, E. B. & Garcia, M. L. Nanostructured lipid carriers for triamcinolone acetonide delivery to the posterior segment of the eye. *Colloids Surf. B Biointerfaces* **88,** 150–157 (2011).

15. Agarwal, R. *et al.* Liposomes in topical ophthalmic drug delivery: an update. *Drug Deliv.* **23,** 1075–1091 (2016).

16. Bochot, A. & Fattal, E. Liposomes for intravitreal drug delivery: a state of the art. *J. Control. Release* **161,** 628–634 (2012).

17. Kaiser, J. M. *et al.* Nanoliposomal minocycline for ocular drug delivery. *Nanomedicine* **9,** 130–140 (2013).

18. Lajavardi, L. *et al.* Downregulation of endotoxin-induced uveitis by intravitreal injection of vasoactive intestinal Peptide encapsulated in liposomes. *Invest. Ophthalmol. Vis. Sci.* **48,** 3230–3238 (2007).

19. Lajavardi, L. *et al.* New formulation of vasoactive intestinal peptide using liposomes in hyaluronic acid gel for uveitis. *J. Control. Release* **139,** 22–30 (2009).

20. Baatz, H., Puchta, J., Reszka, R. & Pleyer, U. Macrophage depletion prevents leukocyte adhesion and disease induction in experimental melanin-protein induced uveitis. *Exp. Eye Res.* **73,** 101–109 (2001).

21. Broekhuyse, R. M., Huitinga, I., Kuhlmann, E. D., Van Rooijen, N. & Winkens, H. J. Differential Effect of Macrophage Depletion on Two Forms of Experimental Uveitis Evoked by Pigment Epithelial Membrane Protein (EAPU), and by Melanin-Protein (EMIU). *Exp. Eye Res.* **65,** 841–848 (1997).

22. Pouvreau, I. *et al.* Effect of macrophage depletion by liposomes containing dichloromethylene-diphosphonate on endotoxin-induced uveitis. *J. Neuroimmunol.* **86,** 171–181 (1998).

23. Puchta, J., Pleyer, U. & Reszka, R. Macrophage depletion inhibits leukocyte recruitment in experimental melanin-induced uveitis (EMIU). *: Zeitschrift der Deutschen …* (2002).

24. Whitcup, S. M. *et al.* Topical liposome-encapsulated FK506 for the treatment of endotoxin-induced uveitis. *Ocul. Immunol. Inflamm.* **6,** 51–56 (1998).

25. Milla, P., Dosio, F. & Cattel, L. PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. *Curr. Drug Metab.* **13,** 105–119 (2012).

26. Metselaar, J. M. *et al.* Liposomal targeting of glucocorticoids to synovial lining cells strongly increases therapeutic benefit in collagen type II arthritis. *Ann. Rheum. Dis.* **63,** 348–353 (2004).

27. Metselaar, J. M., Wauben, M. H. M., Wagenaar-Hilbers, J. P. A., Boerman, O. C. & Storm, G. Complete remission of experimental arthritis by joint targeting of glucocorticoids with long-circulating liposomes. *Arthritis Rheum.* **48,** 2059–2066 (2003).

28. Allijn, I. E. *et al.* Liposome encapsulated berberine treatment attenuates cardiac dysfunction after myocardial infarction. *J. Control. Release* **247,** 127–133 (2017).

29. Hofkens, W., Schelbergen, R., Storm, G., van den Berg, W. B. & van Lent, P. L. Liposomal targeting of prednisolone phosphate to synovial lining macrophages during experimental arthritis inhibits M1 activation but does not favor M2 differentiation. *PLoS One* **8,** e54016 (2013).

30. van den Hoven, J. M. *et al.* Liposomal drug formulations in the treatment of rheumatoid arthritis. *Mol. Pharm.* **8,** 1002–1015 (2011).

31. Lobatto, M. E. *et al.* Pharmaceutical development and preclinical evaluation of a GMP-grade anti-inflammatory nanotherapy. *Nanomedicine* **11,** 1133–1140 (2015).

32. Ghosn, C. R. *et al.* Treatment of experimental anterior and intermediate uveitis by a dexamethasone intravitreal implant. *Invest. Ophthalmol. Vis. Sci.* **52,** 2917–2923 (2011).

33. Nussenblatt, R. B., Palestine, A. G., Chan, C. C. & Roberge, F. Standardization of vitreal

inflammatory activity in intermediate and posterior uveitis. *Ophthalmology* **92,** 467–471 (1985).

34. Bloch-Michel, E. & Nussenblatt, R. B. International Uveitis Study Group recommendations for the evaluation of intraocular inflammatory disease. *Am. J. Ophthalmol.* **103,** 234–235 (1987).

35. Gan, L. *et al.* Self-assembled liquid crystalline nanoparticles as a novel ophthalmic delivery system for dexamethasone: Improving preocular retention and ocular bioavailability. *Int. J. Pharm.* **396,** 179–187 (2010).

36. Hofauer, B., Bas, M., Manour, N. & Knopf, A. Liposomal local therapy as treatment for sicca symptoms in patients with primary Sjögren's syndrome. *HNO* **61,** 921–927 (2013).

37. Böhm, M., Avgitidou, G., El Hassan, E. & Mösges, R. Liposomes: a new non-pharmacological therapy concept for seasonal-allergic-rhinoconjunctivitis. *Eur. Arch. Otorhinolaryngol.* **269,** 495–502 (2012).

38. Díaz-Llopis, M. *et al.* Liposomally-entrapped ganciclovir for the treatment of cytomegalovirus retinitis in AIDS patients. Eperimental toxicity and pharmacokinetics, and clinical trial. *Doc. Ophthalmol.* **82,** 297–305 (1992).

39. Jones, R., 3rd & Rhee, D. J. Corticosteroid-induced ocular hypertension and glaucoma: a brief review and update of the literature. *Curr. Opin. Ophthalmol.* **17,** 163–167 (2006).

40. Clark, A. F. & Wordinger, R. J. The role of steroids in outflow resistance. *Exp. Eye Res.* **88,** 752–759 (2009).

41. Barar, J., Aghanejad, A., Fathi, M. & Omidi, Y. Advanced drug delivery and targeting technologies for the ocular diseases. *Bioimpacts* **6,** 49–67 (2016).

42. Barar, J., Javadzadeh, A. R. & Omidi, Y. Ocular novel drug delivery: impacts of membranes and barriers. *Expert Opin. Drug Deliv.* **5,** 567–581 (2008).

43. Storm, G., Steerenberg, P. A., Emmen, F., van Borssum Waalkes, M. & Crommelin, D. J. Release of doxorubicin from peritoneal macrophages exposed in vivo to doxorubicin-containing liposomes. *Biochim. Biophys. Acta* **965,** 136–145 (1988).

44. Storm, G., Regts, J., Beijnen, J. H. & Roerdink, F. H. Processing of Doxorubicin-Containing Liposomes by Liver Macrophages in Vitro. *J. Liposome Res.* **1,** 195–210 (1989).

5

Characterisation of the inflammatory cytokine and growth factor profile in a rabbit model of proliferative vitreoretinopathy

Chee Wai Wong[1,2,5]
Ning Cheung[1,2,5]
Candice Ho[2]
Veluchamy Barathi[2,5]
Gert Storm[3,6]
Tina T. Wong[1,2,4,5]

1. Singapore National Eye Centre (SNEC), 11 Third Hospital Avenue, Singapore 168751
2. Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore 168751
3. Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands
4. School of Materials Science and Engineering (MSE), Nanyang Technological University, 11 Faculty Avenue, Singapore 639977
5. Ophthalmology and Visual Sciences Academic Clinical Program, Duke NUS Medical School, 8 College Rd, Singapore 169857
6. Dept. Biomaterials Science & Technology (BST), Section Targeted Therapeutics, University of Twente, PO Box 217, 7500 AE Enschede, The Netherlands

Sci Rep. 2019 Oct 28;9(1):15419

Abstract

Purpose: To measure the vitreous levels of pro-inflammatory cytokines and growth factors during the evolution of proliferative vitreoretinopathy (PVR) in a rabbit model.

Methods: PVR was surgically induced in 11 rabbit eyes by vitrectomy, retinotomy, cryotherapy and injection of platelet-rich plasma at baseline. Severity of PVR was assessed on dilated fundal examination with indirect binocular ophthalmoscopy and graded based on the revised experimental PVR classification. Severe PVR was defined as stage 5 or worse. Vitreous concentrations of interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 1 beta (IL-1 β), tumor necrosis factor beta (TNF-β), granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), C reactive protein; (CRP), placental growth factor (PlGF), platelet derived growth factor BB (PDGF-BB), vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang-2) at weeks 2, 3 and 4 were compared to baseline and correlations between the cytokines with PVR severity were assessed.

Results: Four weeks after PVR induction, 5 eyes (45.5%) had developed severe PVR. IL-8 was raised at 2 weeks post PVR induction (1.46±0.48pg/ml vs 0.53±0.25pg/ml, p=0.04) and remained significantly elevated at week 4 (2.6±3.1pg/ml, p=0.03). CRP was significantly raised at week 4 (34.8±12.0pg/ml vs 13.0±13.1pg/ml, p<0.001). Among the growth factors, PDGF-BB was the earliest to show significantly elevated levels, at 3 weeks (50.4±19.0pg/ml vs 6.2±10.1pg/ml) and remained elevated at week 4 (p=0.002), while PlGF (11.2±7.7pg/ml vs 5.3±3.8pg/ml, p=0.002) and Ang2 (13617.0±8170.2pg/ml vs 38593.8±8313.4, p=0.02) were significantly raised at week 4. IFN-γ (p=0.03), PDGF-BB (p=0.02) and VEGF (p=0.02) were significantly associated with PVR severity.

Conclusions: Inflammatory cytokines IL-6, -8, and Ang2 elevation post PVR induction is followed by elevated levels of fibroproliferative growth factors, VEGF and PDGF-BB in the development of PVR. These findings will guide future studies targeting appropriate therapeutic strategies for the treatment of PVR.

Introduction

Proliferative vitreoretinopathy (PVR) is a blinding condition that can occur secondary to penetrating ocular trauma, retinal detachment, or following surgery for retinal detachment repair. In these conditions, a breach in the integrity of the retina introduces macrophages, retinal pigment epithelial cells, glial cells, and fibroblasts into the vitreous, where they proliferate and incite inflammation. This process has been likened to keloidal scar formation, which in the eye can result in massive retinal detachment, scarring and obliteration of vision.[1] PVR is the most common reason for failure of retinal detachment (RD) surgery: anatomical success rates in RD complicated by PVR is only 69-75% compared to 98% in RD without PVR, and visual outcomes of this surgery are worse when complicated by PVR.[2,3] Although surgery is the mainstay of treatment for RD complicated by PVR, multiple surgeries are frequently required to eventually achieve final retinal attachment often with unsatisfactory visual outcomes.[3] In addition, following RD surgery, patients with PVR require twice as many care resources compared to patients without PVR. These resources include not only the economic burden of multiple surgeries but also a longer time spent recovering from surgery and thus away from employment, longer follow up duration and increased patient burden for the hospital, as well as the emotional burden of poor visual outcomes for both patients and their caretakers.[3]

For the past 40 years, many pharmacological agents have shown promising results in animal models of PVR but none have successfully achieved clinical application due to limited efficacy in humans. This failure to translate preclinical success can perhaps be attributed to 2 main reasons. First, there is a lack of clarity in the pathogenesis of PVR. This in turn led to the development of inappropriate animal models that do not reflect the disease process and therefore led to a lack of clinical efficacy for therapeutic agents tested using these models. Second, PVR is a multi-stage disease involving many pathogenic pathways. These pathways can be broadly grouped into inflammation, proliferation and epithelial mesenchymal transition processes. A single agent cannot be expected to be efficacious in all multiple cellular processes that together make up the clinical complication. Instead, a multi-agent

therapeutic strategy directed at the correct targets at the correct time should be the approach of choice for treating such a complex disease.

To help clarify the mechanisms and their temporal relationship in the development of PVR, we conducted a study of changes in cytokine levels following surgically induced PVR in the rabbit. This rabbit model is a surgical model based on human pathogenesis, i.e. PVR following retinal detachment and thus reflects clinically relevant disease. The aim of this study was to compare the levels of pro-inflammatory cytokines and growth factors involved in RPE cell proliferation and epithelial mesenchymal transition at various time points in the evolution of PVR, and to correlate these levels to the severity of PVR.

Materials and methods

Animals

The SingHealth Institute Animal Care and Use Committee (IACUC Singhealth Approval Number 2016/SHS/1256) approved this study. All procedures conducted in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. 11 New Zealand White adult rabbits, with weights of 2-2.5kg were used in this study. Only rabbits with no ocular disease as confirmed on slit lamp examation were included in the study.

Induction of PVR[4,5]

After sterilizing the eye with 5% iodine solution, 23-gauge trans pars plana vitrectomy (Stellaris PC, Bausch and Lomb, Irvine, CA) was performed. Four retinotomies, of 500um (one third disc diameter) in size each were performed with a 41 gauge needle and bleb retinal detachments of 3-4 disc diameters were induced by injection of balanced salt solution at 4 separate sites in the inferior retina. Rabbits received an intravitreal injection, using a 25-gauge needle into the central vitreous, 4mm behind the

limbus of 0.1ml platelet rich plasma (PRP). PRP was prepared from rabbit homologous blood according to the method of Constable *et al*.[6] Pooled arterial blood was collected from the rabbit's ear artery into plastic tubes containing an anticoagulant solution (1 part 3.8% sodium citrate to 9 parts whole blood). This fresh citrated blood was centrifuged at 1,200 rotations per min for 10 min, and the upper third of the supernatant PRP was aspirated. Tobramycin eyedrops were instilled into the eye 4 times a day for 5 days after induction of PVR.

Investigations and examination

The retinal status was examined with an indirect ophthalmoscope through a +20 D fundus lens on days 1, 7, 14, 21 and 28. by two double-masked ophthalmologists (CWW, DC). PVR was graded according to the revised PVR classification:

Revised PVR classification[7]

Stage 0: Normal retina (A)

Stage 1: Surface wrinkling (B)

Stage 2: Mild pucker (C)

Stage 3: Severe pucker (D)

Stage 4: Elevated pucker (E)

Stage 5: Partial retinal detachment (F)

Stage 6: Low detachment (G)

Stage 7: Total detachment (H)

Severe PVR was defined as stage 5 or worse PVR. Fundus photographs were taken with a 45-degree digital retinal camera after pupillary dilation with tropicamide1%, using Canon CR-DGi with Canon EOS 10D SLR backing (Canon Inc, Tokyo, Japan).

Collection of vitreous samples and analysis

Vitreous humor samples were obtained at baseline during the start of the vitrectomy procedure. At day 14 and 21, vitreous humor samples of 0.2ml each were obtained with a 23G needle on a 5ml syringe via

the pars plana. On day 28, eyes were enucleated and the vitreous obtained prior to paraffin fixation of the eye. Vitreous samples were stored at -80 degrees Celsius prior to analysis. The vitreous concentrations of interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 1 beta (IL-1β), tumor necrosis factor beta (TNF-β), granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), C reactive protein; (CRP), placental growth factor (PlGF), platelet derived growth factor BB (PDGF-BB), vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang-2) were determined using the Human multiplex ELISA kit from AYOXXA

Enucleation, euthanasia & pathology procedures

Euthanization was carried out on all rabbits at the end of the 28 day study period with intraperitoneal pentobarbitone (60-150mg/kg). The study eyes were then enucleated.

Histopathology and immunohistochemistry (Table 1)

The procedures performed for histology and immunohistochemistry have been previously described by our group.[8] Eyes were enucleated and fixed in a mixture of 10% neutral buffered formalin solution (Leica Surgipath , Leica Biosystems Richmond, Inc.) for 24 hours. The whole eye were then dissected to anterior and posterior segment prior to dehydration in increasing concentration of ethanol, clearance in xylene, and embedding in paraffin (Leica-Surgipath, Leica Biosystems Richmond, Inc.) Four-micron sections were cut with a rotary microtome (RM2255, Leica Biosystems Nussloch GmbH, Germany) and collected on POLYSINE^{TM} microscope glass slides (Gerhard Menzel, Thermo Fisher Scientific, Newington, CT). The sections were dried in an oven of 37^{o}C for at least 24 hour. To prepare the sections for histopathological and immunohistochemical examination, the sections were heated on a 60^{o}C heat plate, deparaffinized in xylene and rehydrated in decreasing concentration of ethanol. A standard procedure for Hematoxylin and Eosin (H&E) was performed. A light microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides and images were captured.

In parallel, immunofluorescence staining was performed. Heat-induced antigen retrieval was performed by incubating sections in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) for 20 minutes at 95-100^{o}C. The sections were then cooled down in sodium citrate buffer for 20 minutes in RT

and washed three times for 5 minutes each with 1X PBS. Non-specific sites were blocked with blocking solution of 5% bovine serum albumin (BSA) in 0.1% Triton X-100 and 1XPBS for 1 hour at room temperature in a humidified chamber. The slides were then rinsed briefly with 1X PBS. A specific primary antibody shown in Table 1 was applied and incubated overnight at 4^{o}C in a humidified chamber prepared in blocking solution. After washing twice with 1XPBS and once with 1X PBS with 0.1% tween for 10 minutes each, Alexa Fluro® 488 – conjugated fluorescein-labeled secondary antibody shown in Table 1 (Invitrogen- Molecular Probes, Eugene, OR) was applied at a concentration of 1:1000 in blocking solution and incubated for 90 minutes at RT. The slides were then washed twice with 1XPBS and once with 1X PBS with 0.1% tween for 5 minutes each, the slides were mounted on the slides with Prolong Diamond Anti-fade DAPI5 Mounting Media (Invitrogen- Molecular Probes, Eugene, OR) to visualize cell nucleic. For negative controls, primary antibody was omitted.

A fluorescence microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides and images were captured. Experiments were repeated in duplicates for the antibody.

Table 1:Antibodies used for immunohistochemistry

Antibody	**Catalog No.**	**Company**	**Concentration**
Smooth muscle actin	710487	Thermo fisher Scientific	1:200
GFAP GA5	14-9892-82	Thermo fisher Scientific	1:200
Vimentin	MA511883	Thermo fisher Scientific	1:200
Alexa Fluor 488 goat anti−mouse IgG (H+L)	A11001	Invitrogen. Life Technologies (Invitrogen, Eugene, OR)	1:1000

Statistical analysis

Statistical analysis was performed with Stata 13.0 (Stata Corporation, College Station, TX).

Continuous data were presented as mean ± standard deviation (SD). Cytokine levels were compared

with the paired t test while proportions were analysed with the chi square test. The *P*-value for trend across time from PVR induction were calculated, and multivariable analysis was performed to assess associations of cytokine levels with PVR severity, using ordinal logistic regression adjusted for time from PVR induction. A two-tailed p value of < 0.05 was considered statistically significant.

Results

PVR severity

Figure 1 shows the distribution of PVR severity across the study period. At 2 weeks after PVR induction, most eyes (n=7, 63.6%) had developed at least stage 1 PVR and none had severe PVR. There is an increase in number of eyes with severe PVR (Figure 2A) at week 3 (n=3, 27.2%)). Four weeks after PVR induction, 5 eyes (45.5%) had developed severe PVR.

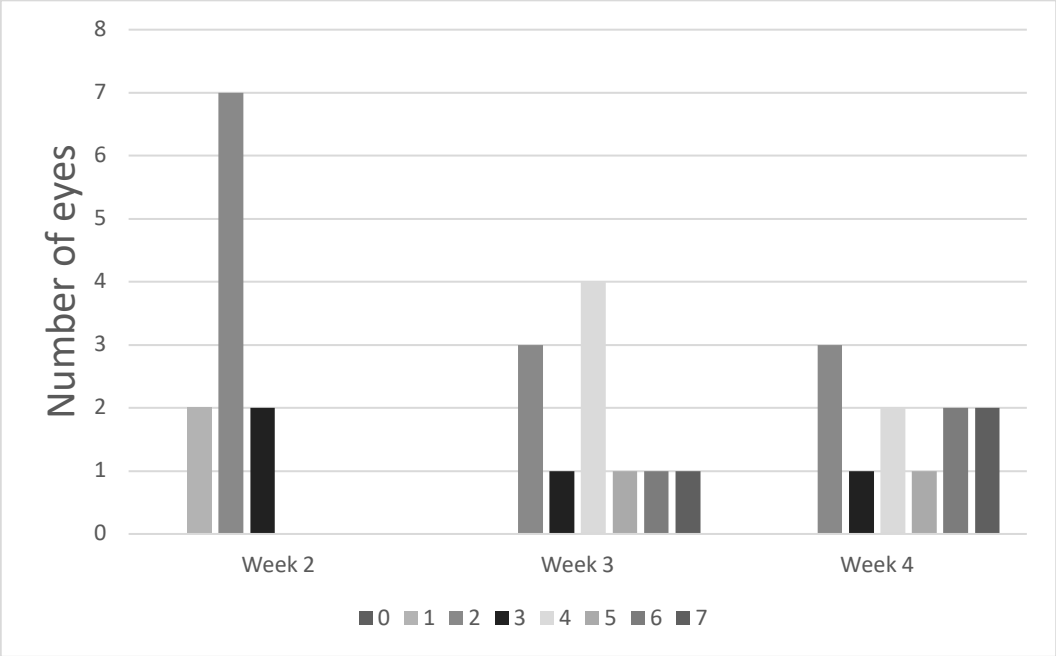

Figure 1: Number of eyes in each stage of PVR severity at different time intervals after PVR induction.

Cytokine and growth factor levels

Table 2 shows the mean cytokine levels at baseline, weeks 2,3 and 4 post PVR induction. IL-8 was significantly raised at 2 weeks post PVR induction (1.46±0.48pg/ml vs 0.53±0.25pg/ml, p=0.04) and remained significantly elevated at week 4 (2.6±3.1pg/ml, p=0.03). CRP was significantly raised at week 4 (34.8±12.0pg/ml vs 13.0±13.1pg/ml, p<0.001).

PDGF-BB was the earliest to show significantly elevated levels, at 3 weeks (50.4±19.0pg/ml vs 6.2±10.1pg/ml) and remained elevated at week 4 (p=0.002). PlGF (11.2±7.7pg/ml vs 5.3±3.8pg/ml, p=0.002) and Ang2 (13617.0±8170.2pg/ml vs 38593.8±8313.4, p=0.02) were significantly raised at week 4.

IL-6, IL-8, CRP, PDGF-BB, PGF, VEGF and Ang2 all showed significant trend for elevation over the 4 weeks experimental duration.

Association of cytokine and growth factor levels with PVR severity

Table 3 shows the association of cytokine levels with severity of PVR. IFN-γ (p=0.03), CRP (p=0.001), PDGF-BB (p<0.001) and VEGF (p=0.002) were significantly associated with PVR severity. After adjusting for time from PVR induction, IFN-γ (p=0.03), PDGF-BB (p=0.02) and VEGF (p=0.02) remained significantly associated with PVR severity.

Table 2: Mean cytokine and growth factor concentrations in the vitreous from baseline to week 4

Cytokine levels, pg/ml	Baseline	Week 2	P*	Week 3	P*	Week 4	P*	P trend
IL-6	0.39±0.25	0.59±0.24	0.38	0.67±0.23	0.32	0.72±0.57	0.06	0.008
IL-8	0.53±0.25	1.46±0.48	0.04	11.5±17.0	0.22	2.6±3.1	0.03	<0.001
IL-1β	0.20±0.05	0.24±0.06	0.58	0.46±0.13	0.11	0.37±1.90	0.12	0.13
TNF-β	2.1±0.79	3.23±1.05	0.47	3.59±0.54	0.29	0.37±0.13	0.12	0.71
GM-CSF	0.29±0.05	0.33±0.06	1.00	0.39±0.03	0.29	0.29±0.07	0.89	0.75
IFN-γ	3.8±1.77	5.07±1.53	1.00	9.28±1.20	0.29	6.08±2.65	0.14	0.21
CRP	13.0±13.1	30.7±12.1	0.22	35.9±9.8	0.07	34.8±12.0	0.0008	0.001
PlGF	5.3±3.8	9.0±3.8	0.66	13.2±5.2	0.05	11.2±7.7	0.02	0.02
PDGF-BB	6.2±10.1	26.2±8.4	0.32	50.4±19.0	0.0009	34.6±27.4	0.002	0.001
VEGF	215.1±145.5	311.9±188.0	0.58	1875.5±1356.8	0.11	1251.5±1876.0	0.13	0.02
Ang2	13617.0±8170.2	106464.6±57182.56	0.18	24064.2±10409.9	0.47	38593.8±8313.4	0.02	0.007

*compared to baseline

Abbreviations:

IL-6, interleukin 6; IL-8, interleukin 8; IL-1β, interleukin 1 beta; TNF-β, tumor necrosis factor beta; GM-CSF, granulocyte macrophage colony stimulating factor; IFN-γ , interferon gamma; CRP, C reactive protein; PlGF, placental growth factor; PDGF-BB, platelet derived growth factor BB; VEGF, vascular endothelial growth factor; Ang2, angiopoietin 2.

Table 3: Association of cytokines and growth factors with PVR severity

Molecular factor	unadjusted OR	95% CI	p	adjusted OR	95% CI	p
IL-6	2.66	0.38 - 18.63	0.32	0.30	0.03 - 3.17	0.32
IL-8	1.04	0.97 - 1.11	0.24	1.03	0.97 - 1.10	0.37
IL-1β	1.33	0.65 - 2.71	0.44	1.05	0.43 - 2.57	0.92
TNF-β	1.02	0.92 - 1.13	0.69	0.99	0.87 - 1.13	0.93
GM-CSF	257.87	0.07 - 94000	0.19	2300000	0.64 - 8.41e+12	0.06
IFN-γ	1.34	1.03 - 1.75	0.03	1.68	1.05 - 2.69	0.03
CRP	1.09	1.04 - 1.15	0.001	1.03	0.96 - 1.10	0.39
PlGF	1.00	0.99 - 1.01	1.00	1.01	0.99 - 1.02	0.40
PDGF-BB	1.06	1.03- 1.09	<0.001	1.04	1.01 - 1.08	0.02
VEGF	1.000109	1.000039 - 1.000179	0.002	1.000088	1.000017 - 1.000159	0.02

Ang2	1.000004	.9999948 - 1.000014	0.38	1.00	1.00 - 1.00	0.96

Abbreviations:

IL-6, interleukin 6; IL-8, interleukin 8; IL-1β, interleukin 1 beta; TNF-β, tumor necrosis factor beta; GM-CSF, granulocyte macrophage colony stimulating factor; IFN-γ , interferon gamma; CRP, C reactive protein; PlGF, placental growth factor; PDGF-BB, platelet derived growth factor BB; VEGF, vascular endothelial growth factor; Ang2, angiopoietin 2.

Histology and immunohistochemistry

H&E staining confirmed traction on the inner retina with folding of the outer retina in detached retina (Figure 2B and C). Vimentin, a protein expressed by de-differentiated RPE cells, mesenchymal cells, Müller cells and other glial cells, was observed both in the subretinal space (Fig. 2D). Alpha smooth muscle actin (α-SMA), a marker for myofibroblasts derived predominantly from dedifferentiated RPE cells, was observed in both epiretinal membranes and in the subretinal space (Fig. 2E and H). Epiretinal membranes also stained positively for Glial fibrillary acid protein (GFAP), a marker of glial cells (Figure 2F). Figure 3 shows the corresponding images for an eye with stage 2 PVR (mild pucker).

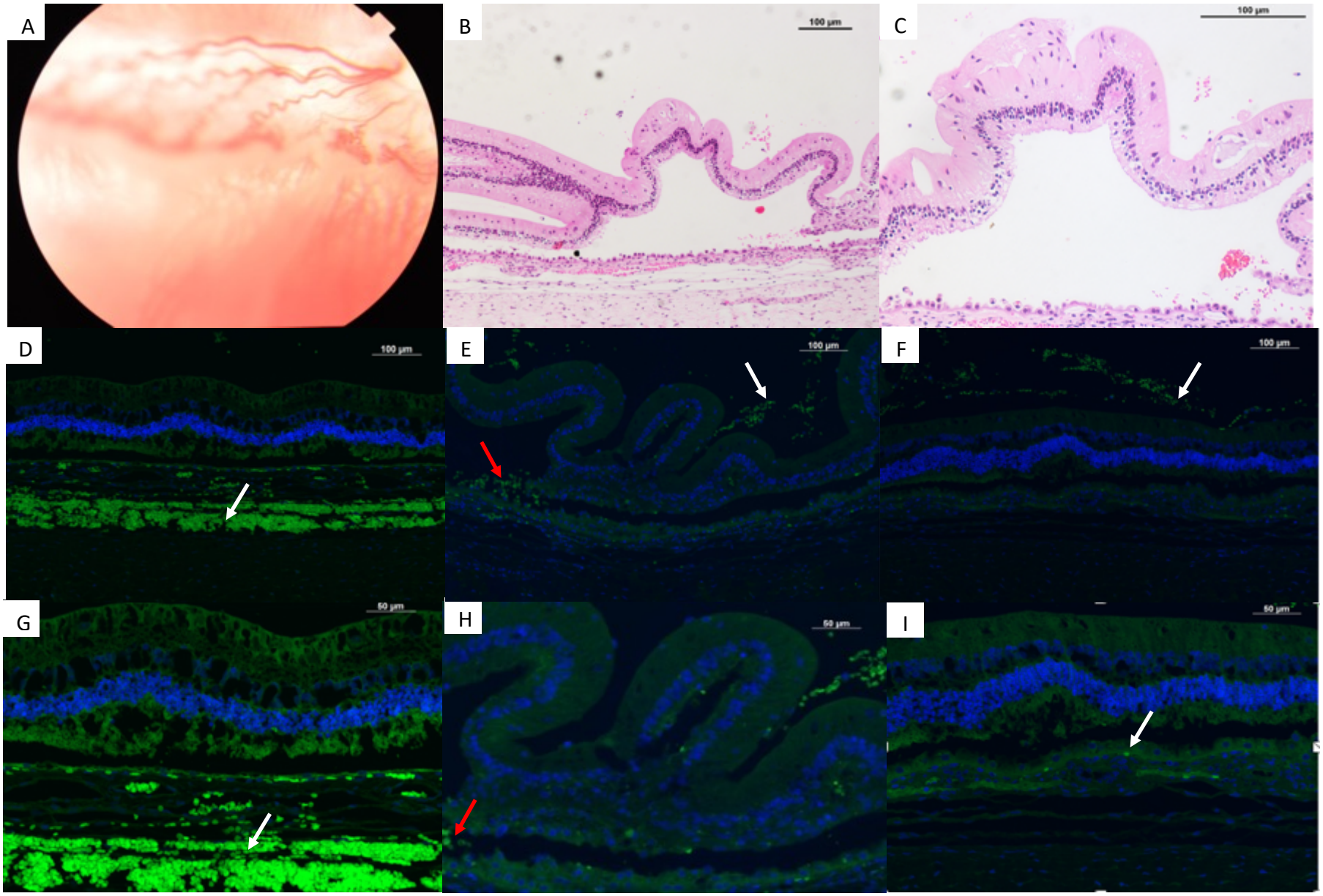
A
B
100 µm
C
100 µm
D
100 µm
E
100 µm
F
100 µm
G
50 µm
H
50 µm
I
50 µm

Figure 2: Fundus photo, H&E staining and immunohistochemical staining of a rabbit eye with severe PVR. (A) Fundus photo shows partial detachment of the retina with folding of the detached retina. Detachment and folding of the retina can be observed in detail on H&E staining at 10X (B) and 20X (C) magnification. Epiretinal membranes (white arrows) stained positively with alpha smooth muscle actin (E) and glial fibrillary acid protein (F), demonstrating the presence of glial and mesenchymal elements in these membranes. Vimentin staining was present within the subretinal space (D and G, white arrow) and smooth muscle actin staining was present in subretinal membranes as well (E and H, red arrows). G, H and I show vimentin, alpha smooth muscle actin and glial fibrillary acid protein staining at 20X magnification.

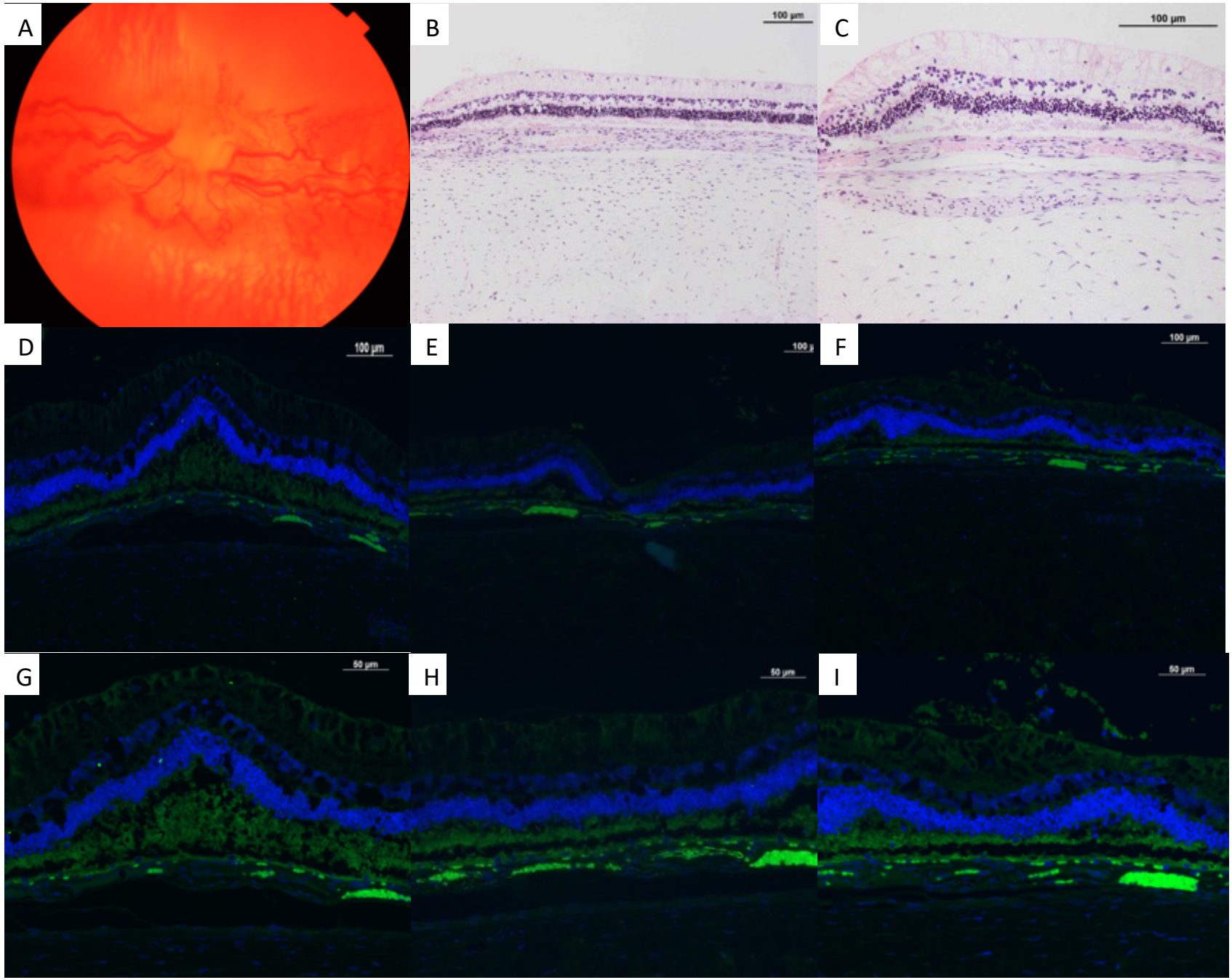
A
B
100 µm
C
100 µm
D
100 µm
E
F
100 µm
G
50 µm
H
50 µm
I
50 µm

Figure 3: Fundus photo, H&E staining and immunohistochemical staining of a rabbit eye with Stage 2 PVR. (A) Fundus photo shows mild puckering of the medullary wings with distortion of blood vessels. There is no retinal detachment on H&E staining at 10X (B) and 20X (C) magnification. There is no staining of retinal surface membranes with vimentin (D,G), alpha smooth muscle actin (E,H) and glial fibrillary acid protein (F,I) at 10X or at 20X magnification.

Discussion

PVR is a complex disease with multiple stages in its evolution, involving many pathogenic pathways. To develop an effective therapeutic strategy, it is important to understand which pathways are involved and how to target them at the appropriate timing. Many models of PVR have been described over the years.[9] Most of the earlier models involved injection of fibroblasts into the vitreous cavity in an attempt to recreate the fibrosis seen in PVR. We now know that external fibroblasts are not involved in the PVR process.[10] To more accurately model the emergence of human disease, we chose an experimental model that mimicked the development of PVR after surgery, by inducing retinal detachment, release of RPE cells into the vitreous cavity, simulating a pro-inflammatory environment with cryotherapy and the injection of platelet rich plasma, avoiding the injection of any non-native cells. With this model, we found that PVR began as early as 2 weeks after surgery, with about 50% of eyes developing severe PVR with retinal detachment at week 4. Our PVR model was similar to that described by Goldaracena et al., in which they performed vitrectomy, retinotomy, cryotherapy and PRP injection. In contrast with our results, they found 100% rate of severe PVR with retinal detachment at week 4, with clinically apparent signs of PVR appearing at week 2 to 3.[5] Several variables in surgical factors could have contributed to this observed difference, including the extent of cryotherapy, size of retinotomies, amount of vitreous removed and number of RPE cells liberated into the vitreous cavity. To further optimize our surgical model, considerations are being made for controlling such factors.

In brief, the postulated pathogenic processes that occur during retinal detachment that lead to PVR are as follows[10]: 1. breakdown of the blood retinal barrier allowing microglia and macrophages to migrate into the subretinal space and the vitreous cavity where they release inflammatory cytokines, 2. release of RPE cells into the vitreous cavity, where they are stimulated by growth factors produced by a variety of cells including Müller cells to survive and proliferate, and 3. RPE cells then undergo mesenchymal transformation (epithelial mesenchymal transition, EMT) into fibroblast-like cells that

then form contractile membranes on the surface of the retina, within the retina, and in the subretinal space. These PVR membranes may subsequently redetach the retina. It is clear from this sequence of events that a pro-inflammatory environment is a crucial first step for the initiation of the PVR disease process. Several inflammatory cytokines have been associated with PVR, including IL-6, IL-8, IL-10, IL-1β and interferon γ.[11-16] Our findings demonstrate that inflammation (as represented by elevation of IL-8) spikes within the first 2 weeks and continues to persist up to 4 weeks after induction of the PVR process. CRP and IFN-γ were significantly associated with PVR severity, suggesting that inflammation not only incites the PVR process, but also perpetuates its severity. Interestingly, the association of IFN-γ with PVR severity was independent of the duration post PVR induction. IFN-γ has been shown to cause RPE dysfunction by increasing the expression of the long noncoding RNA (lncRNA) BANCR. BANCR expression has been shown to elicit EMT like changes in cancer cells as well as ARPE-19 cells.[17]

Survival and proliferation of RPE cells are the next important step in the pathogenesis of PVR. Tumor protein 53 (TP53) suppression by activation of PDGF receptor α (PDGFR α)
is a key event, allowing these cells to resist apoptosis and enhance proliferation. PDGFRα can be directly activated by PDGFs or indirectly by non-PDGFs. PDGFs have consistently been found to be elevated in both animal models of PVR as well as in human PVR.[18,19] However, it is the non-PDGF activation of PDGFRα that appears to be the major pathway of TP53 suppression as it circumvents the receptor downregulation mediated by PDGFs, allowing perpetual activation of PDGFRα.[18] In particular, VEGF promotes the non-PDGF pathway of activating PDGFRα by antagonizing PDGF-mediated dimerization of PDGFRs[20,21], and anti-VEGF agents have been shown to completely suppress PVR development in animal models.[21,22] We found a significant trend for elevation of VEGF levels over 4 weeks in this study, and VEGF was also significantly associated with PVR severity independent of time from PVR induction. PlGF is a member of the VEGF family, bearing remarkable similarity in its three dimensional structure with VEGF isoform A.[23] We observed elevated levels of PlGF at 4 weeks post PVR induction, but how PlGF is involved in PVR pathogenesis is currently unknown.

Ang2, another member of the VEGF family, is a multifaceted cytokine involved in the regulation of angiogenesis and inflammation.[24] Increased levels of Ang2 has been found in vitreous samples from eyes with retinal detachment and have been suggested to contribute to PVR development[25], but there are no studies to date describing an association with PVR. We found significantly elevated levels of Ang2 at week 4 post PVR induction. Interestingly, Ang2 has been implicated in lung cancer metastasis by increasing EMT.[26] Future research should investigate the possible role of Ang2 in promoting PVR via the EMT pathway.

There are limitations to our study. The sample size is small and may not be adequately powered to study small differences in cytokine and growth factor levels. This limitation is somewhat mitigated by vitreous sampling over many time points. There are many other cytokines that have been reported to be associated with PVR that we did not analyse in this study. However, we have chosen the most consistently reported cytokines and those that have been shown to play an important role in human PVR pathogenesis. Lastly, the study period of 1 month may not have been sufficient for PVR development in some of the study eyes. Nevertheless, having study eyes of different PVR severity did allow us to characterise the cytokine composition at the various stages of PVR development in the rabbit eye, which is the main objective of this study.

In conclusion, our study in a rabbit PVR model demonstrates intense inflammation beginning rapidly in the first 2 weeks after PVR induction which continues to be elevated up till 4 weeks post PVR induction. Shortly after the inflammatory phase, the growth factors IFN-γ , VEGF, PDGF-BB, PlGF and Ang2 likely support the survival, proliferation and EMT of RPE cells. The findings regarding Ang2 warrant further studies to better understand the role of this cytokine in PVR development. The findings presented here will guide future studies aiming to study inflammatory cytokines and growth factors as potential therapeutic targets for the treatment of PVR.

References

1. Newsome DA, Rodrigues MM, Machemer R. Human massive periretinal proliferation. In vitro characteristics of cellular components. *Archives of ophthalmology.* 1981;99(5):873-880.

2. Wong CW, Wong WL, Yeo IY, et al. Trends and factors related to outcomes for primary rhegmatogenous retinal detachment surgery in a large asian tertiary eye center. *Retina.* 2014;34(4):684-692.

3. Patel NN, Bunce C, Asaria RH, Charteris DG. Resources involved in managing retinal detachment complicated by proliferative vitreoretinopathy. *Retina.* 2004;24(6):883-887.

4. Lei H, Rheaume MA, Velez G, Mukai S, Kazlauskas A. Expression of PDGFRalpha is a determinant of the PVR potential of ARPE19 cells. *Investigative ophthalmology & visual science.* 2011;52(9):5016-5021.

5. Goldaracena MB, Garcia-Layana A, Pastor JC, Saornil MA, de la Fuente F, Gayoso MJ. The role of retinotomy in an experimental rabbit model of proliferative vitreoretinopathy. *Curr Eye Res.* 1997;16(5):422-427.

6. Constable IJ, Oguri M, Chesney CM, Swann DA, Colman RW. Platelet-induced vitreous membrane formation. *Invest Ophthalmol.* 1973;12(9):680-685.

7. Hida T, Chandler DB, Sheta SM. Classification of the stages of proliferative vitreoretinopathy in a refined experimental model in the rabbit eye. *Graefes Arch Clin Exp Ophthalmol.* 1987;225(4):303-307.

8. Wong CW, Czarny B, Metselaar JM, et al. Evaluation of subconjunctival liposomal steroids for the treatment of experimental uveitis. *Sci Rep.* 2018;8(1):6604.

9. Agrawal RN, He S, Spee C, Cui JZ, Ryan SJ, Hinton DR. In vivo models of proliferative vitreoretinopathy. *Nat Protoc.* 2007;2(1):67-77.

10. Pastor JC, Rojas J, Pastor-Idoate S, Di Lauro S, Gonzalez-Buendia L, Delgado-Tirado S. Proliferative vitreoretinopathy: A new concept of disease pathogenesis and practical consequences. *Prog Retin Eye Res.* 2016;51:125-155.

11. Banerjee S, Savant V, Scott RA, Curnow SJ, Wallace GR, Murray PI. Multiplex bead analysis of vitreous humor of patients with vitreoretinal disorders. *Investigative ophthalmology & visual science.* 2007;48(5):2203-2207.

12. Hui Y, Shi Y, Zhang X, Yang K, Yu C. [TNF-alpha, IL-8 and IL-6 in the early inflammatory stage of experimental PVR model induced by macrophages]. *Zhonghua Yan Ke Za Zhi.* 1999;35(2):140-143.

13. Asaria RH, Kon CH, Bunce C, et al. Silicone oil concentrates fibrogenic growth factors in the retro-oil fluid. *The British journal of ophthalmology.* 2004;88(11):1439-1442.

14. La Heij EC, van de Waarenburg MP, Blaauwgeers HG, et al. Basic fibroblast growth factor, glutamine synthetase, and interleukin-6 in vitreous fluid from eyes with retinal detachment complicated by proliferative vitreoretinopathy. *Am J Ophthalmol.* 2002;134(3):367-375.

15. Liou GI, Pakalnis VA, Matragoon S, et al. HGF regulation of RPE proliferation in an IL-1beta/retinal hole-induced rabbit model of PVR. *Mol Vis.* 2002;8:494-501.

16. Elner SG, Elner VM, Jaffe GJ, Stuart A, Kunkel SL, Strieter RM. Cytokines in proliferative diabetic retinopathy and proliferative vitreoretinopathy. *Curr Eye Res.* 1995;14(11):1045-1053.

17. Kutty RK, Samuel W, Duncan T, et al. Proinflammatory cytokine interferon-gamma increases the expression of BANCR, a long non-coding RNA, in retinal pigment epithelial cells. *Cytokine.* 2018;104:147-150.

18. Lei H, Velez G, Kazlauskas A. Pathological signaling via platelet-derived growth factor receptor {alpha} involves chronic activation of Akt and suppression of p53. *Mol Cell Biol.* 2011;31(9):1788-1799.

19. Pennock S, Rheaume MA, Mukai S, Kazlauskas A. A novel strategy to develop therapeutic approaches to prevent proliferative vitreoretinopathy. *Am J Pathol.* 2011;179(6):2931-2940.

20. Pennock S, Kazlauskas A. Vascular endothelial growth factor A competitively inhibits platelet-derived growth factor (PDGF)-dependent activation of PDGF receptor and subsequent signaling events and cellular responses. *Mol Cell Biol.* 2012;32(10):1955-1966.

21. Pennock S, Haddock LJ, Mukai S, Kazlauskas A. Vascular endothelial growth factor acts primarily via platelet-derived growth factor receptor alpha to promote proliferative vitreoretinopathy. *Am J Pathol.* 2014;184(11):3052-3068.

22. Pennock S, Kim D, Mukai S, et al. Ranibizumab is a potential prophylaxis for proliferative vitreoretinopathy, a nonangiogenic blinding disease. *Am J Pathol.* 2013;182(5):1659-1670.

23. De Falco S. The discovery of placenta growth factor and its biological activity. *Exp Mol Med.* 2012;44(1):1-9.

24. Scholz A, Plate KH, Reiss Y. Angiopoietin-2: a multifaceted cytokine that functions in both angiogenesis and inflammation. *Ann N Y Acad Sci.* 2015;1347:45-51.

25. Loukovaara S, Lehti K, Robciuc A, et al. Increased intravitreal angiopoietin-2 levels associated with rhegmatogenous retinal detachment. *Graefes Arch Clin Exp Ophthalmol.* 2014;252(6):881-888.

26. Dong Z, Chen J, Yang X, et al. Ang-2 promotes lung cancer metastasis by increasing epithelial-mesenchymal transition. *Oncotarget.* 2018;9(16):12705-12717.

6

Endogenous or exogenous retinal pigment epithelial cells: A comparison of 2 experimental animal models of proliferative vitreoretinopathy

Chee Wai Wong[1,2,3]
Joanna Marie Fianza Busoy[2]
Ning Cheung[1,2,3]
Veluchamy Amutha Barathi[2]
Gert Storm[4,5]
Tina T. Wong[1,2,3]

1. Singapore National Eye Centre (SNEC), 11 Third Hospital Avenue, Singapore 168751
2. Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore 168751
3. Duke-NUS Graduate Medical School, 8 College Rd, Singapore 169857
4. Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands.
5. Department of Experimental Molecular Imaging, University Clinic and Helmholtz Institute for Biomedical Engineering, RWTH Aachen University, Aachen 52074, Germany

Transl Vis Sci Technol. 2020 Aug 31;9(9):46

Abstract

*Purpos*e: Proliferative vitreoretinopathy (PVR) is a blinding condition that can occur following ocular penetrating injury and retinal detachment. To develop effective therapeutics for PVR, it is imperative to establish an animal model that is reproducible, closest in anatomy to the human eye and most representative of the human disease. We compared 2 *in-vivo* models of PVR in minipig eyes to assess reproducibility and consistency.

Methods: Six minipigs underwent PVR induction with procedure A and 6 underwent procedure B. In both procedures, PVR was induced with vitrectomy, bleb retinal detachment, retinotomy, and injection of platelet rich plasma. In procedure A, retinal pigment cells (RPE) were harvested from cadaveric pig eyes and injected at the end of surgery. In procedure B, native RPE cells were released into the vitreous cavity by creating a RPE detachment and scraping of the RPE layer. PVR severity was graded on fundoscopic examination with a modified Silicone Study classification system for PVR. Severe PVR was defined as stage 2-5.

Results: Three (50%) and 5 (83.3%) eyes developed re-detachment of the retina from severe PVR in procedure A and B respectively (p=0.55). Median PVR stage was higher in eyes that underwent procedure B than A, although the difference was not statistically significant (2.5 vs 1.5, p=0.26)

Conclusions: This new model utilising native RPE cells achieved a high consistency in inducing severe PVR in the minipig.

Introduction

Proliferative vitreoretinopathy (PVR) is a blinding condition that can occur secondary to penetrating ocular trauma, retinal detachment, or following surgery for retinal detachment repair. In these conditions, a breach in the integrity of the retina introduces macrophages, retinal pigment epithelial cells, glial cells, and fibroblasts into the vitreous, where they proliferate and incite inflammation. This process has been likened to keloidal scar formation, but in the eye, can result in massive retinal detachment, scarring and obliteration of vision.[1] PVR is the most common reason for failure of retinal detachment (RD) surgery: anatomical success rates in RD complicated by PVR is only 69-75% compared to 98% in RD without PVR, and visual outcomes of this surgery are worse when complicated by PVR.[2, 3] Although surgery is the mainstay of treatment for PVR, multiple surgeries are frequently required to eventually achieve final retinal attachment with unsatisfactory visual outcomes.[3] In addition, following surgery for retinal detachment, patients with PVR require twice as many resources to care for compared to patients without PVR.[3]

For the past 40 years, many pharmacologic agents have shown promising results in animal models of PVR, but none have made it to routine clinical application due to limited efficacy in humans. This failure to translate preclinical success can be attributed to the fact that PVR is a multifactorial disease, parts of which are still unclear. These animal models may each be simulating particular pathways in the pathogenesis of PVR and in doing so, may have involved pathways that are not present in human PVR pathogenesis or missed critical pathways altogether.[4]

To develop effective therapeutics for PVR, it is imperative to establish an animal model that is most representative of the human disease. In our previous study, we attempted to create a rabbit model of PVR by combining vitrectomy, retinotomy, cryotherapy and injection of platelet rich plasma. However, this model produced inconsistent results: out of 11 eyes, only 5 developed clinically

significant PVR with retinal detachment.[5] In most of these eyes, clinical observation was hindered by the development of varying degrees of cataract.

An ideal animal model of PVR should have an anatomy and physiology that is as close to the human eye as possible. Minipigs have several advantages as an animal model of ocular disease and is increasingly used to as a substitute for nonhuman primates due to its close similarity to the human eye for the following reasons. First, the size of the minipig eye and lens most closely approximate that of a human eye. Second, the intraocular distribution of melanin is most similar to the human eye. This is important when considering pharmacokinetic studies as drug compounds often bind to melanin. Third, the vitreous humor of the mini pig has similar composition to the human vitreous thus making it ideal for testing retinal therapeutics. Fourth, the smaller crystalline lens of the minipig eye reduces the likelihood of iatrogenic cataract formation following surgical intervention that may interfere with retinal examination. Finally, the retina of the minipig is very similar to that of a human. The retinal vasculature is holangiotic and there is no tapetum lucidum in the minipig eye. Although there is no macula, minipig eyes have a visual streak containing a high density of cone photoreceptors, like the human fovea.

The first major step towards creating a PVR model that was closest to the human disease was made by García-Layana et al in 1997. Their group was the first to switch animal species to the pig model to capitalize on its anatomical similarities with the human eye. In addition, they demonstrated that the injection of external cells was not necessary to induce PVR, which was revolutionary at that time when most believed that fibroblast injection was essential for PVR to develop, a thinking that made models of that time very distant from the clinical reality.[6] Their work effectively demonstrated that endogenous cells, together with growth factors, plasma components and their interaction with platelet derived growth factor can induce PVR that was similar to the human disease. We aim to build upon this work and to leverage on the advancement of our understanding in PVR pathogenesis since then to develop a minipig model of PVR that is consistent and similar to the human disease. In this study, we

compare two surgical models of PVR in the minipig eye, using exogenous or native retinal pigment epithelial (RPE) cells[7] to induce PVR disease process.

Methods

The SingHealth Institute Animal Care and Use Committee (IACUC, Singhealth Approval Number 2017/SHS/1328) gave approval for this study. All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve eyes of 12 Gottingen minipigs were used for this study. Age range was 8-14 months and average weight was 20kg. The minipigs were anesthetized with intraperitoneal injection of ketamine hydrochloride (35- 50mg/kg) and Xylazil (5- 10mg/kg) prior to induction of PVR. Topical anaesthesia (proparacaine hydrochloride 0.5%) was applied and pupils were dilated with tropicamide 1%. Six pigs underwent procedure A and 6 underwent procedure B as described below.

Platelet rich plasma preparation

In both procedures, platelet rich plasma (PRP) were prepared from minipig homologous blood 30 minutes prior to surgery, according to the method of Constable *et al*.[8] Arterial blood was collected from the minipig's ear artery into citrate blood tubes (1 part 3.8% sodium citrate to 9 parts whole blood). The blood was centrifuged at 1,200 rotations per min for 10 min, and the upper third of the supernatant PRP was aspirated. Platelet counts were performed on an automated Coulter counter to achieve 600,000 platelets per cubic millimetre of 0.1ml of PRP were injected into the vitreous cavity at the end of surgery.

Procedure A

RPE isolation

Retinal pigment epithelial (RPE) cells were isolated from cadaveric pig eyes using the methodology as described by Sonoda et al.[9] RPE cells were harvested 30 minutes before use to ensure viability of the injected cells. The RPE cell clusters were kept on ice in Dulbecco's modified Eagle's medium (DMEM) just before use.

Induction of PVR

PVR was induced based on a modified methodology of Umazume et al.[7] Three-port valved 25-gauge pars plana vitrectomy (Constellation; Alcon) was performed, 3mm from the limbus, taking care to avoid traumatising the lens. First, posterior vitreous detachment was induced by suction, followed by core vitrectomy and shaving of the peripheral vitreous. Then, inferior retinal detachment was induced by injecting balanced salt solution (Alcon Laboratories, Fort Worth, TX) into the subretinal space with a 39-gauge angled cannula. Subretinal injections were performed in the inferior retinal hemisphere, at least 1-disc diameter away from the optic disc, avoiding any visible blood vessels. A retinotomy of 3-disc diameters was created within the inferiorly detached retina using the vitrector. If bleeding occurred, infusion pressure was increased to 60mmHg to achieve haemostasis. Finally, RPE cells (8×10^4 cells) in 0.1 mL of Dulbecco's modified Eagle's medium (DMEM) and platelet rich plasma (PRP) were injected into the vitreous cavity. The modifications from the methodology of Umazume et al[7] were: 1. We used 25-gauge vitrectomy instead of 20-gauge; 2. We created an inferior retinal detachment instead of a total retinal detachment; 3. We created retinotomies of 3 -disc diameters and injected platelet rich plasma, steps that were not performed in the previously described methodology. These modifications were made to minimise the differences between Procedures A and B, such that the procedures would differ mainly on the basis of exogenous RPE injection vs release of endogenous RPE cells.

Procedure B

With the exception of exogenous RPE cell isolation, the initial steps are similar to procedure A. Briefly, 3-port valved 25-gauge pars plana vitrectomy (Constellation; Alcon) was performed, 3mm from the

limbus. Posterior vitreous detachment was induced followed by core vitrectomy and shaving of the peripheral vitreous. The following steps differentiate the 2 procedures: RPE detachment was induced by injecting balanced salt solution (Alcon Laboratories, Fort Worth, TX) into the sub-RPE space, using a 39-gauge angled cannula. Inducing a RPE detachment allowed easier access to the sub RPE space, as compared to inducing a retinal detachment in Procedure A. An RPE detachment is distinct from a retinal detachment and can be detected on direct visualisation. A retinotomy of 3-disc diameters was created within this area of RPE detachment with the vitrector. A 39 gauge angled cannula was then advanced into the sub-RPE space to scrape the RPE and dislodge these cells into the vitreous cavity (Figure 1 and Supplementary video). The release of RPE cells into the vitreous cavity simulates the process which occurs in human PVR, in which RPE cells escape from the subretinal space into the vitreous cavity through a retinal break. A 90% fluid air exchange was performed to disrupt any remaining vitreous and to extend the PVD to the peripheral retina so as to allow RPE cells to settle onto the inferior retinal surface. Air was used instead of gas as it is short-lasting and would avoid the problems of post-operative formation of gas cataract or poor visualisation.

For both procedures, topical Tobramycin was administered 4 times a day for 5 days after induction of PVR.

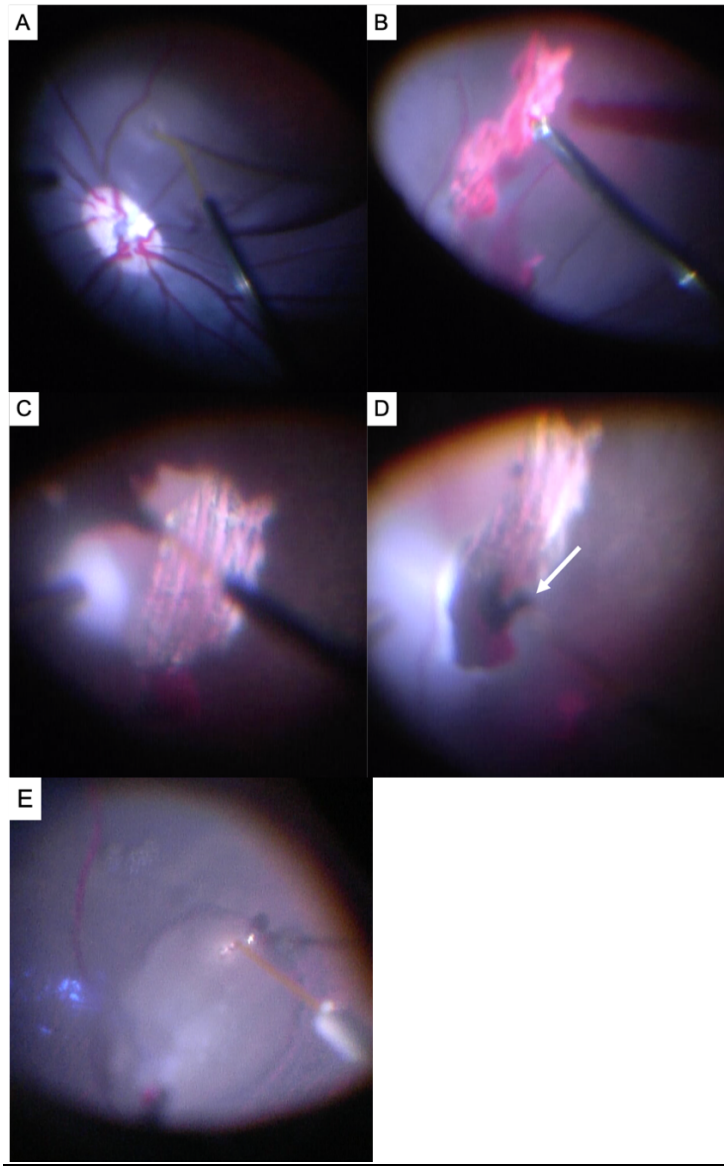

Figure 1: Intraoperative photos of the surgical steps. (A) After posterior vitreous detachment and vitrectomy, a 39-gauge angled cannula is advanced into the sub retinal pigment epithelial space (RPE). Balanced salt solution is slowly infused into the sub RPE space to create a RPE detachment. (B) A large retinotomy is created with the vitrector in a radial fashion to avoid transecting large blood vessels. (C) The RPE is scraped with an angled cannula to dislodge it from the retina. (D) Large clumps of RPE cells (white arrow) are released into the vitreous cavity. (E) Induction of retinal detachment in Procedure A.

Investigations and examination

The retina was examined with an indirect ophthalmoscope through a +20 D fundus lens on days 1, 7, 14, 21 and 28 by two double-masked ophthalmologists. The choice of PVR classification is crucial to evaluating an animal model in terms of how representative the model is with the human disease. Classification systems for animal models of PVR were mainly developed for rabbits, including the Fastenberg classification[10] that was designed for intact vitreous PVR models, and the revised PVR classification[11] which was proposed for a vitrectomised model/cell intravitreal injection model. In this study, we opted to use a modified version of the Silicon Study Classification System, a classification of human PVR, that was also employed by Umazume's group[7] in their description of a swine model of PVR:

Grade 0: Normal retina, retinal or vitreous pigment clumps

Grade 1: Inner retinal wrinkling

Grade 2: Retinal detachment, 1 quadrant (1-3 clock hours)

Grade 3: Retinal detachment, 2 quadrants (4-6 clock hours)

Grade 4: Retinal detachment, 3 quadrants (7-9 clock hours)

Grade 5: Retinal detachment, 4 quadrants (10-12 clock hours)

Severe PVR was defined as retinal re-detachment i.e. stage 2-5. Fundus photographs and optical coherence tomography (OCT) was performed using a spectral domain (SD)-OCT system (Spectralis, Heidelberg Engineering Inc. Germany). At the end of study, minipigs were euthanized with intraperitoneal pentobarbitone (60- 150mg/kg) and study eyes were enucleated.

Collection of vitreous samples and analysis

Vitreous humor samples of at least 0.2ml were obtained at the start of the vitrectomy procedure with the vitrector attached to a 3 ml syringe. On day 28, eyes were enucleated and the vitreous obtained prior to paraffin fixation of the eye. Vitreous samples were stored at -80 degrees Celsius prior to

analysis. The vitreous concentrations of interleukin 6 (IL-6), C reactive protein; (CRP), platelet derived growth factor BB (PDGF-BB) and vascular endothelial growth factor (VEGF) were determined using the Human multiplex ELISA kit from AYOXXA.

Histopathology and Immunohistochemistry

Mini pig eyes were fixed in a mixture of 10% neutral buffered formalin solution (Leica Surgipath, Leica Biosystems Richmond, Inc.) for 24 hours. The whole eye was then dissected to anterior and posterior segment prior to dehydration in increasing concentration of ethanol, clearance in xylene, and embedding in paraffin (Leica-Surgipath, Leica Biosystems Richmond, Inc.) Four-micron sections were cut with a rotary microtome (RM2255, Leica Biosystems Nussloch GmbH, Germany) and collected on POLYSINE^{TM} microscope glass slides (Gerhard Menzel, Thermo Fisher Scientific, Newington, CT). The sections were dried in an oven of 37^{o}C for at least 24 hours. The sections were then heated on a 60^{o}C heat plate, deparaffinized in xylene and rehydrated in decreasing concentration of ethanol in preparation for staining. A standard procedure for Hematoxylin and Eosin (H&E) was performed. A light microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides and images were captured.

For immunofluorescence staining, heat-induced antigen retrieval was performed by incubating sections in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) for 20 minutes at 95-100^{o}C. The sections were then cooled down in sodium citrate buffer for 20 minutes in RT and washed three times for 5 minutes each with 1X PBS. Non-specific sites were blocked with blocking solution of 5% bovine serum albumin (BSA) in 0.1% Triton X-100 and 1XPBS for 1 hour at room temperature in a humidified chamber. The slides were then rinsed briefly with 1X PBS. A specific primary antibody shown in Table 1 was applied and incubated overnight at 4^{o}C in a humidified chamber prepared in blocking solution. After washing twice with 1XPBS and once with 1X PBS with 0.1% tween for 10 minutes each, Alexa Fluro® 488 – conjugated fluorescein-labeled secondary antibody shown in Table 1 (Invitrogen- Molecular Probes, Eugene, OR) was applied at a

concentration of 1:1000 in blocking solution and incubated for 90 minutes at RT. The slides were then washed twice with 1XPBS and once with 1X PBS with 0.1% tween for 5 minutes each, the slides were mounted on the slides with Prolong Diamond Anti-fade DAPI5 Mounting Media (Invitrogen-Molecular Probes, Eugene, OR) to visualize cell nuclei. For negative controls, primary antibody was omitted.

A fluorescence microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides and images were captured. Experiments were repeated in duplicates for the antibody.

Table 1: Antibodies used in immunohistochemistry

Antibody	**Catalog No.**	**Company**	**Concentration**
Smooth muscle actin	710487	Thermo fisher Scientific	1:200
Cytokeratin	MA513156	Thermo fisher Scientific	1:200

Statistics

The Chi-square analysis (2-sided) was used for to compare proportion of eyes with severe PVR between the 2 groups. Student T test was used to compare mean PVR stage and cytokine levels between the groups. Statistical significance was set at p=0.05 and Stata version 16.0 was used for the analysis.

Results

PVR staging

On day 28, with procedure A, a total of 3 eyes (50%) developed re-detachment of the retina due to severe PVR (Figure 2 and 3). 3 eyes developed PVR stage 1, 1 developed PVR stage 2 and 2 developed PVR stage 5. With procedure B, 5 eyes (83.3%) had retinal redetachment due to severe PVR. One eye developed stage 1 PVR, 2 developed stage 2, 1 developed stage 3 and 2 stage 5 PVR. There was no statistically significant difference in median PVR stage (1.5 vs 2.5, p=0.26), or proportion of severe PVR (p=0.55).

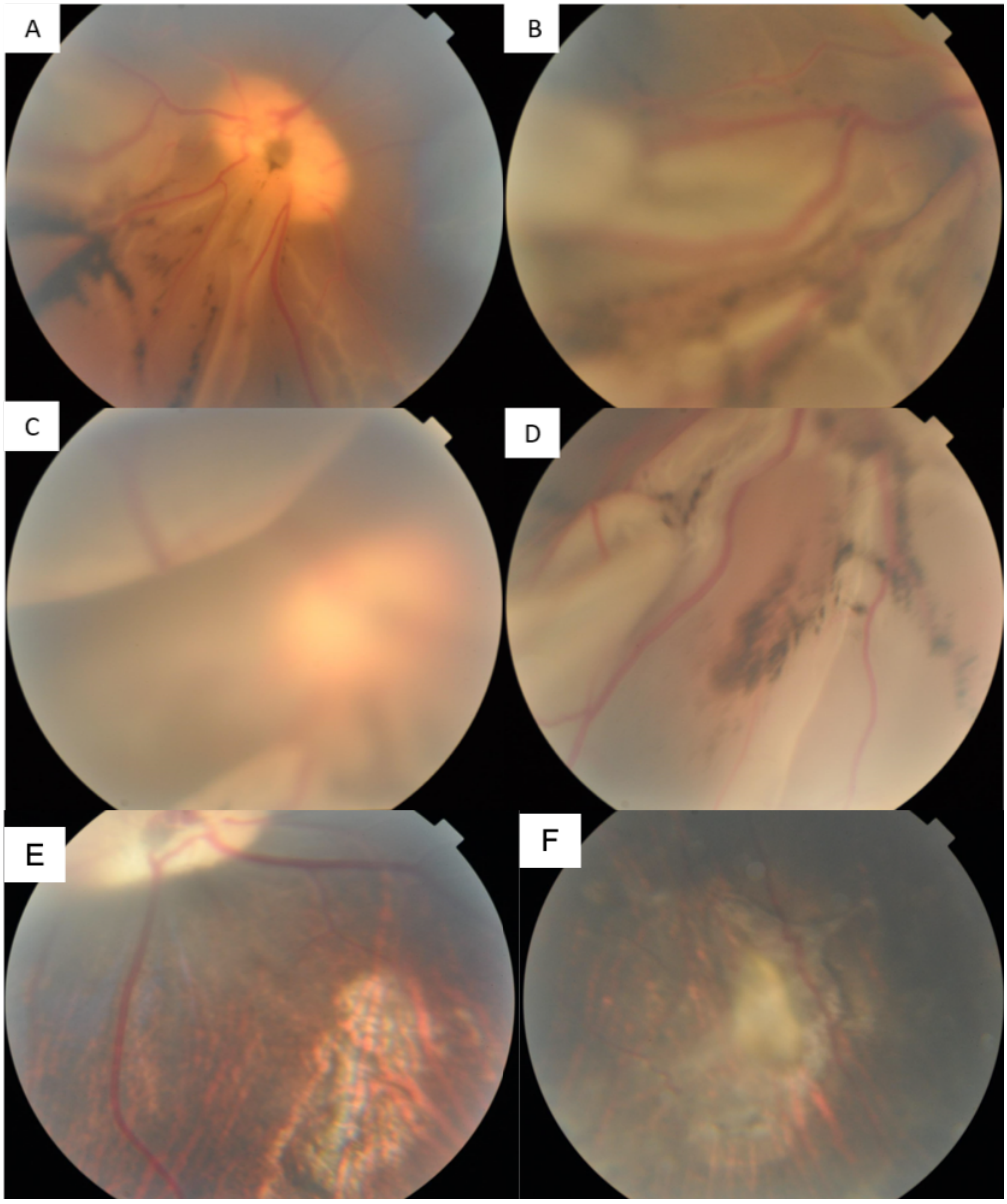

Figure 2: Fundus photographs at Day 14 (A and B) and Day 28 (C and D) of an eye with stage 5 PVR. On Day 14, there is detachment of the inferior and temporal retina (A). Retinal folds and deposits of retinal pigment epithelial (RPE) cells can be seen on the retinal surface (B). On Day 28, the retinal detachment had progressed to involve the entire retina (C). Fixed retinal folds associated with RPE cells can be observed in the inferior retina. (E) An eye with reattachment of the retina. RPE atrophy is seen surrounding a fibrotic retinal scar (F).

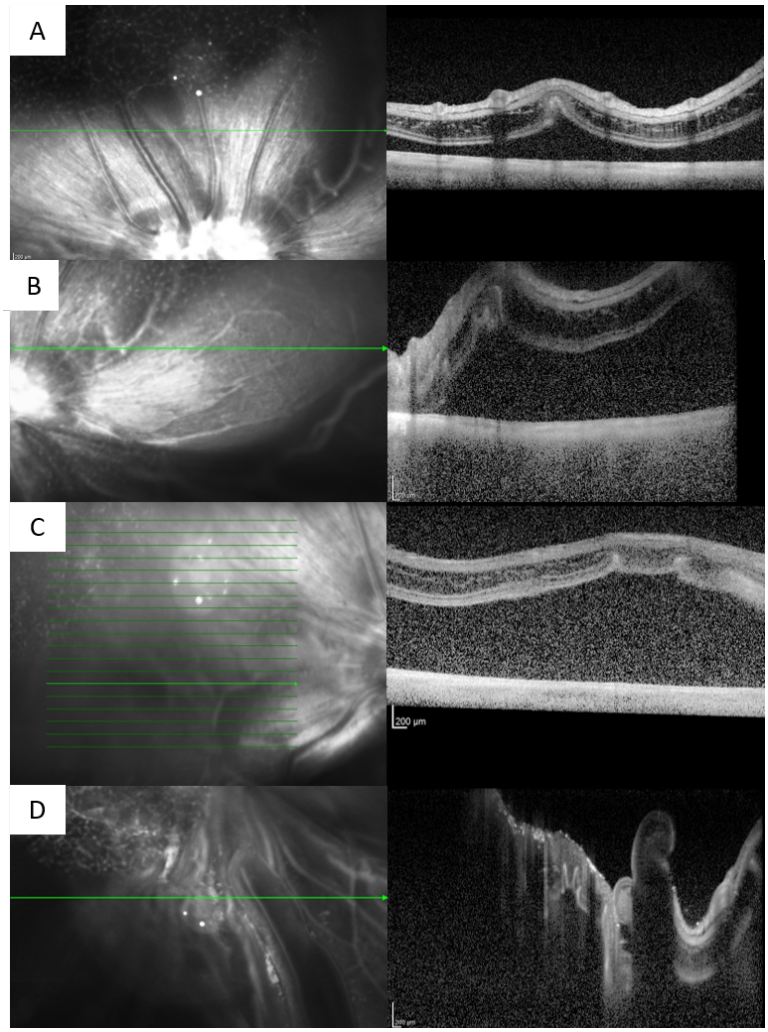

Figure 3: Optical coherence tomographic scans of the same eye on Day 28. Green lines on the red free images (left) represent the scanned segments. These scans confirm the presence of retinal detachment in the superior (A), temporal (B), nasal (C), and inferior retina (D). Folds in the inferior retina (D) can be seen. These folds result from traction exerted by PVR membranes.

Cytokine and growth factor levels

Mean levels of inflammatory cytokines and growth factors were, in general, higher in group B than group (table 1), including CRP, IL-6, PDGF-BB and VEGF-A, but did not reach statistical significance.

Table 1: Cytokine and growth factor levels at week 4

Cytokine (pg/ml)	Procedure A	Procedure B	p
IL-6	1.0 ± 1.5	1.7 ± 1.9	0.53
CRP	27.0 ± 33.3	50.1 ± 20.4	0.19
PDGF-BB	5.2 ± 5.9	20.0 ± 16.3	0.21
VEGF-A	783.9 ± 848.2	895.6 ± 554.3	0.87

Abbreviations: IL-6, interleukin 6; CRP, C-reactive protein; PDGF-BB, platelet derived growth factor BB; VEGF-A, vascular endothelial growth factor isoform A.

Histology and immunohistochemistry

H&E staining demonstrated folding of the inner retina, characteristic of PVR (Figure 4A &B). Alpha smooth muscle actin, a marker for myofibroblasts derived mainly from dedifferentiated RPE cells, was observed on epiretinal membranes (Figure 4C). Cytokeratin positive spindle cells can be seen within fibrocellular membranes associated with these retinal folds (Figure 4D).

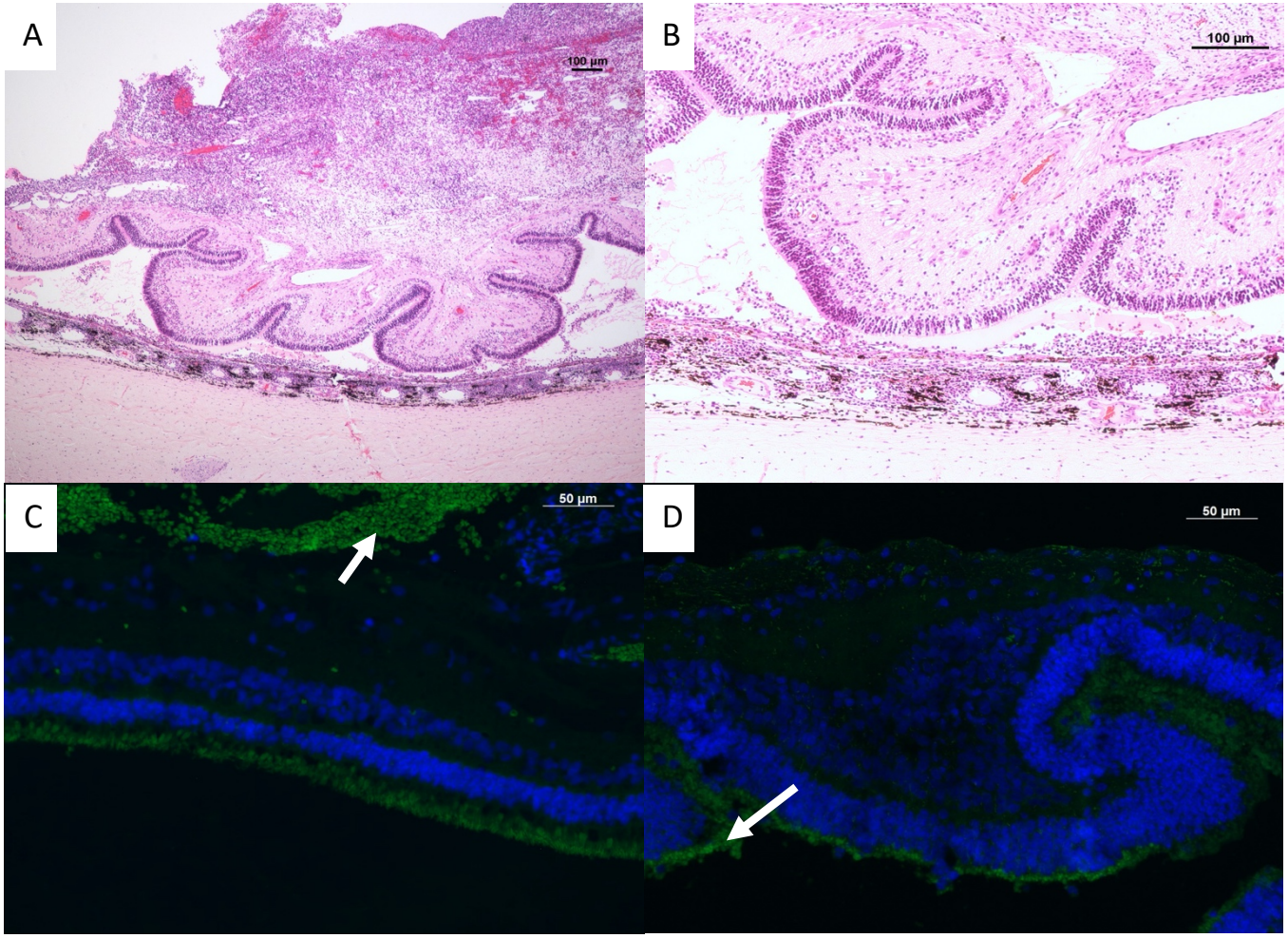

Figure 4: Histopathological staining of the retina in an eye with PVR. (A) H&E stain at 10x magnification demonstrating folding of the detached retina. (B) The same H&E stain at 20x magnification. (C) Immunohistochemical staining showing the presence of alpha smooth muscle actin on epiretinal membranes (arrow) and positive cytokeratin staining (D, white arrow) on subretinal membranes.

Discussion

This is the first study comparing 2 surgical animal models of PVR in the minipig: injection of exogenous RPE cells (Procedure A) versus in situ release of endogenous RPE cells (Procedure B). Procedure B was designed to more closely follow and reflect the pathogenic events in clinical PVR. We observed higher rates of severe PVR, as well as higher concentration of inflammatory cytokines and growth factors associated with PVR with this model, although statistical significance was not reached. A larger study is required to validate these findings.

Different animal species have been used to model PVR each their own pros and cons.[12] For example, rabbit models have the advantage of large vitreous volume and relative ease of manipulation with less risk of damage to the lens and retina compared to smaller animals like the rat. However, their retinal structure including blood vessels and nerve fiber distribution differ from that of humans, complicating direct comparison to the human disease in anatomic and pathologic terms. Rodent models are less commonly employed. While murine species are relatively easier to modify genetically, their large crystalline lens and small vitreous volume severely limit the feasibility of surgical manipulation and fundus examinations.[13, 14] Pig models are rarely used, but increasingly recognised as an ideal substitute for non-human primate models.[6, 7] Their eyes are similar in size to the human eye, their retinae are holangiotic like the human eye, and they have a cone-enriched area centralis which is similar to the human fovea. In this study, we show that PVR that closely follow the pathogenesis of the human disease, i.e. development of fibrotic membranes and tractional retinal detachment following exposure of RPE cells to the vitreous cavity, can be successfully induced in minipig eyes.

Most animal models of PVR do not replicate the pathogenic processes in the human disease. Such models rely on the addition of cells or growth factors associated with the pathogenesis of PVR and may or may not include other interventions to disrupt the vitreous such as with gas injection or vitrectomy.[4] In vivo models in which PVR is induced by intravitreal injection of fibroblasts[15], RPE

cells[7, 16-25], or macrophages[26] introduce large quantities of exogenous cells that do not naturally occur even in the disease state. More importantly, they do not account for key steps in PVR development, such as cellular survival, epithelial mesenchymal transformation and proliferation.[4] Therapeutic agents that are seemingly efficacious in these models may be affecting the injected cells directly rather than inhibiting the endogenous PVR cascade, resulting in falsely promising results. In particular, the fibroblast injection model is inherently flawed because dermal, corneal, or conjunctival fibroblasts are not involved in the pathogenesis of human PVR.

Injection models utilising cultured RPE cells and macrophages are more relevant to human disease.[13, 26] However, the macrophage injection model does not expose RPE cells, which are thought to have a critical role in the development of human PVR.[4] In addition to RPE cells, glial elements such as microglia and muller cells play an equally important role in promoting retinal remodeling, leading to retinal shortening within the neurosensory retina while interacting with macrophages and RPE cells in the subretinal space to form subretinal membranes.[4] The relationship between macrophages and cells from the neuroretina i.e. RPE and glial cells, warrants further investigation. Exogenous RPE cells (Table 2) are useful in animal models of PVR because it is difficult to release endogenous RPE cells in sufficient quantity to trigger the PVR process.[12] Umazume et al described a porcine model of PVR in which injection of cadaveric porcine RPE cells successfully induced severe PVR in 14 out of 14 eyes after by 14 days.[7] However, exogenous RPE cell injections have a few issues, namely the difficulty of keeping these cells viable, risk of infection, and the possibility that these exogenous cells may trigger an immune mediated rejection response.[16] The excessive inflammation induced by the rejection response may detract from the actual PVR process and over-estimate the treatment effect of anti-inflammatory therapeutics on PVR. To avoid these problems, we have developed a model of PVR using exclusively endogenous RPE cells. This is possible in the surgical model we described by inducing a RPE detachment and then accessing the RPE through a retinotomy. With this technique, we were able to consistently release a large quantity of RPE cells into the vitreous cavity. Our observation of a higher re-detachment rate and higher concentration of growth factors related to the later proliferative stage of PVR in Procedure B may suggest the

availability of a larger quantity of free floating and viable endogenous RPE cells in the vitreous cavity, compared to Procedure A where the number of viable exogenous RPE cells may be much lower.

Table 2: Summary of in vivo PVR models utilising retinal pigment epithelial cells.

PVR model	Year	Animal	Summary of procedure	Reported PVR induction rate
Pure injection models				
Autologous RPE cell injection[23]	1981	Rabbit	Injection of autologous RPE cells harvested from enucleated fellow eye	86%
Homologous RPE cell injection[19]	1982	Rabbit	Injection of rabbit RPE without vitrectomy	100%
Heterologous RPE cell injection[16, 18, 25]	1987	Rabbit	Injection of human, bovine, or rat RPE cells without vitrectomy	75%
Homologous RPE cells + PDGF-BB injection[20]	2015	Rabbit	Pars plana removal of 0.2ml vitreous, injection of RPE cells and PDGF-BB	100%
Vitreous compression +injection models				
Gas compression + homologous RPE injection[21, 22]	2002	Rabbit	Perfluorocarbon or sulfur hexafluoride gas injection followed by injection of rabbit RPE cells 7–10 days later	72.7%
Gas compression vitrectomy + homologous RPE cell +PDGF BB injection[17]	2007	Rabbit	Perfluoropropane gas injection followed by gas fluid exchange, injection of rabbit RPE cells and PDGF BB.	Most animals
Mechanical Vitrectomy + injection models				

Vitrectomy + artificial RD + RPE cells injection[7, 24]	2012	Swine	Pars plana vitrectomy followed by subretinal injection to induce retinal detachment followed by intravitreal injection of porcine RPE cells	100%

Abbreviations: PVR, proliferative vitreoretinopathy; RPE, retinal pigment epithelial; PDGF-BB, platelet derived growth factor isoform BB; RD, retinal detachment.

The dispase injection model described by Frenzel et al is an interesting model in which an intravitreal injection of dispase in the rabbit eye was sufficient to induce PVR in all eyes.[27] Dispase cleaved basement membrane, allowing RPE cells to be released into the vitreous cavity without the need for a retinal break. This allowed PVR induction in a relatively inexpensive, technically easy way, eliminating the use of surgical equipment and introduction of exogenous cells. However, a major problem with this model was the formation of cataract and zonular dehiscence, presumably due to the effect of dispase on type IV collagen in the lens capsule. This was demonstrated by Kralinger et al, who found a reproducibility of 87% in PVR induction but 90% of eyes developed severe cataract and lens luxation occurred in 47% of these eyes.[28] In this model, dispase was not washed out of the eye, raising the question of whether the PVR process was triggered as a result of a toxic reaction to dispase.

Injection of PRP simulates the situation in human PVR whereby patients with vitreous haemorrhage and retinal detachment concurrently tend to be at high risk of PVR.[6, 29] The increased risk of PVR arises from the high concentrations of growth factors within the vitreous cavity that results in a conducive environment for survival and EMT of liberated RPE cells within the vitreous. However, injecting whole blood into the vitreous cavity will obstruct the visualisation of the retina. Injecting PRP retains the necessary growth factors while avoiding the problem of poor visualization and inaccurate PVR classification.

There are some limitations to our model. First, we did not find a statistically significant difference between the 2 models in the proportion of eyes with severe PVR. This is likely due to the small sample size, but even with these small numbers, we demonstrate that the use of endogenous cells was at least as reproducible for PVR induction as using exogenous cells, with the added advantage of eliminating the time consuming and expensive step of harvesting RPE cells from cadaveric eyes. Second, surgical models are relatively expensive, requiring equipment for vitrectomy, surgical microscope as well as surgical expertise. It is technically difficult to operate on a pig's eye, and there

is a learning curve to creating a RPE detachment. However, considering the cost of Phase 1 or 2 human clinical trials, it is far more cost effective to evaluate potential therapeutics in an accurate preclinical disease model. Third, scraping of RPE cells may cause more trauma to the retina than performing a simple retinal detachment, but this is not visually evident in terms of retinal tearing or increased bleeding. It is also difficult to quantify if either procedure may cause more inflammation, because the possible additional trauma induced by scraping RPE cells may or may not be more than the inflammation caused by a possible rejection of exogenous RPE cells. Our study was designed to assess exogenous and endogenous RPE cell release models and not to determine if one procedure was more aggressive than the other. Fourth, we performed an air fluid exchange in procedure B, while this step was not performed in the previously described procedure A that we compared to. Fluid air exchange is a common procedure performed during vitrectomy in human patients for various indications. It is short-lasting and does not generally cause gas cataract, even in human patients with clear crystalline lenses like a long acting gas would. We did not notice the formation of gas cataract in any of the eyes that underwent Procedure B. In conclusion, we demonstrate a consistent model of PVR in the minipig that can be surgically induced using native RPE cells. This may be a suitable model for both understanding the pathogenesis of PVR and for testing of novel therapeutics to treat PVR.

1. Newsome DA, Rodrigues MM, Machemer R. Human massive periretinal proliferation. In vitro characteristics of cellular components. *Archives of ophthalmology* 1981;99:873-880.
2. Wong CW, Wong WL, Yeo IY, et al. Trends and factors related to outcomes for primary rhegmatogenous retinal detachment surgery in a large asian tertiary eye center. *Retina* 2014;34:684-692.
3. Patel NN, Bunce C, Asaria RH, Charteris DG. Resources involved in managing retinal detachment complicated by proliferative vitreoretinopathy. *Retina* 2004;24:883-887.
4. Pastor JC, Rojas J, Pastor-Idoate S, Di Lauro S, Gonzalez-Buendia L, Delgado-Tirado S. Proliferative vitreoretinopathy: A new concept of disease pathogenesis and practical consequences. *Prog Retin Eye Res* 2016;51:125-155.
5. Wong CW, Cheung N, Ho C, Barathi V, Storm G, Wong TT. Characterisation of the inflammatory cytokine and growth factor profile in a rabbit model of proliferative vitreoretinopathy. *Sci Rep* 2019;9:15419.
6. Garcia-Layana A, Pastor JC, Saornil MA, Gonzalez G. Porcine model of proliferative vitreoretinopathy with platelets. *Curr Eye Res* 1997;16:556-563.
7. Umazume K, Barak Y, McDonald K, Liu L, Kaplan HJ, Tamiya S. Proliferative vitreoretinopathy in the Swine-a new model. *Invest Ophthalmol Vis Sci* 2012;53:4910-4916.
8. Constable IJ, Oguri M, Chesney CM, Swann DA, Colman RW. Platelet-induced vitreous membrane formation. *Invest Ophthalmol* 1973;12:680-685.
9. Sonoda S, Spee C, Barron E, Ryan SJ, Kannan R, Hinton DR. A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. *Nat Protoc* 2009;4:662-673.
10. Fastenberg DM, Diddie KR, Dorey K, Ryan SJ. The role of cellular proliferation in an experimental model of massive periretinal proliferation. *Am J Ophthalmol* 1982;93:565-572.
11. Hida T, Chandler DB, Sheta SM. Classification of the stages of proliferative vitreoretinopathy in a refined experimental model in the rabbit eye. *Graefes Arch Clin Exp Ophthalmol* 1987;225:303-307.

12. Hou H, Nudleman E, Weinreb RN. Animal Models of Proliferative Vitreoretinopathy and Their Use in Pharmaceutical Investigations. *Ophthalmic Res* 2018;60:195-204.
13. Lin ML, Li YP, Li ZR, Lin JX, Zhou XL, Liang D. Macrophages acquire fibroblast characteristics in a rat model of proliferative vitreoretinopathy. *Ophthalmic Res* 2011;45:180-190.
14. Miller W, Makova KD, Nekrutenko A, Hardison RC. Comparative genomics. *Annu Rev Genomics Hum Genet* 2004;5:15-56.
15. Algvere P, Kock E. Experimental Fibroplasia in the rabbit vitreous. Retinal detachment induced by autologous fibroblasts. *Albrecht Von Graefes Arch Klin Exp Ophthalmol* 1976;199:215-222.
16. Wong CA, Potter MJ, Cui JZ, et al. Induction of proliferative vitreoretinopathy by a unique line of human retinal pigment epithelial cells. *Can J Ophthalmol* 2002;37:211-220.
17. Agrawal RN, He S, Spee C, Cui JZ, Ryan SJ, Hinton DR. In vivo models of proliferative vitreoretinopathy. *Nat Protoc* 2007;2:67-77.
18. Borhani H, Peyman GA, Rahimy MH, Thompson H. Suppression of experimental proliferative vitreoretinopathy by sustained intraocular delivery of 5-FU. *Int Ophthalmol* 1995;19:43-49.
19. Fastenberg DM, Diddie KR, Sorgente N, Ryan SJ. A comparison of different cellular inocula in an experimental model of massive periretinal proliferation. *Am J Ophthalmol* 1982;93:559-564.
20. Ishikawa K, He S, Terasaki H, et al. Resveratrol inhibits epithelial-mesenchymal transition of retinal pigment epithelium and development of proliferative vitreoretinopathy. *Sci Rep* 2015;5:16386.
21. Kuo HK, Chen YH, Wu PC, et al. Attenuated glial reaction in experimental proliferative vitreoretinopathy treated with liposomal doxorubicin. *Invest Ophthalmol Vis Sci* 2012;53:3167-3174.
22. Lee JJ, Park JK, Kim YT, et al. Effect of 2'-benzoyl-oxycinnamaldehyde on RPE cells in vitro and in an experimental proliferative vitreoretinopathy model. *Invest Ophthalmol Vis Sci* 2002;43:3117-3124.

23. Radtke ND, Tano Y, Chandler D, Machemer R. Simulation of massive periretinal proliferation by autotransplantation of retinal pigment epithelial cells in rabbits. *Am J Ophthalmol* 1981;91:76-87.

24. Umazume K, Liu L, Scott PA, et al. Inhibition of PVR with a tyrosine kinase inhibitor, dasatinib, in the swine. *Invest Ophthalmol Vis Sci* 2013;54:1150-1159.

25. Weinsieder A, Radtke ND, Currier GJ, Caple S, Presley E, Weinsieder G. Bovine RPE heterotransplants simulate proliferative vitreoretinopathy (PVR) in rabbits. *Metab Pediatr Syst Ophthalmol (1985)* 1987;10:2-8.

26. Hui YN, Goodnight R, Sorgente N, Ryan SJ. Fibrovascular proliferation and retinal detachment after intravitreal injection of activated macrophages in the rabbit eye. *Am J Ophthalmol* 1989;108:176-184.

27. Frenzel EM, Neely KA, Walsh AW, Cameron JD, Gregerson DS. A new model of proliferative vitreoretinopathy. *Invest Ophthalmol Vis Sci* 1998;39:2157-2164.

28. Kralinger MT, Kieselbach GF, Voigt M, et al. Experimental model for proliferative vitreoretinopathy by intravitreal dispase: limited by zonulolysis and cataract. *Ophthalmologica* 2006;220:211-216.

29. Pinon RM, Pastor JC, Saornil MA, et al. Intravitreal and subretinal proliferation induced by platelet-rich plasma injection in rabbits. *Curr Eye Res* 1992;11:1047-1055.

7

Liposome drug delivery system: Targeted and sustained delivery of steroid to mitigate the severity of proliferative vitreoretinopathy in a minipig model

Chee Wai Wong[1,2,3]
Joanna Marie Fianza Busoy[2]
Danny Cheung[1,2,3]
Shaun Sim[1,2]
Candice Ho[2]
Amutha Barathi Veluchamy[2]
Gert Storm[3,6]
Tina T. Wong[1,2,3]

1. Singapore National Eye Centre (SNEC), 11 Third Hospital Avenue, Singapore 168751
2. Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore 168751
3. Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands.
4. School of Materials Science and Engineering (MSE), Nanyang Technological University, 11 Faculty Avenue, Singapore 639977
5. Lee Kong Chian school of medicine (LKCmedicine), Nanyang Technological University, 11 Mandalay Road, Singapore 308232
6. Department of Experimental Molecular Imaging, University Clinic and Helmholtz Institute for Biomedical Engineering, RWTH Aachen University, Aachen 52074, Germany

Submission in progress

Abstract

Aim: To investigate the efficacy of a small sized liposomal prednisolone phosphate preparation as targeted treatment to mitigate the severity of proliferative vitreoretinopathy (PVR) in an experimental minipig model of PVR.

Methods: Twelve eyes of 6 minipigs underwent PVR induction surgically. Intravitreal injection of liposomal prednisolone phosphate was performed in right eyes, while left eyes served as controls. PVR severity was graded on fundoscopic examination using a modified version of the Silicon Study Classification System. Severe PVR was defined as grade 2-5 on this classification, and the proportion of eyes with retinal detachment from severe PVR was compared between treatment and control groups.

Results: On day 28, 5 eyes (83.3%) in the control group were observed to have severe PVR. Within the control group, PVR grade 1 (Figure 1) was observed in 1 (16.7%) eye, grade 2 in 1 (16.7%) eye, grade 3 in 2 (33.3%) eyes and grade 5 (Figure 1) in 2 (33.3%) eyes. Within the treatment group, only 1 (16.7%) eye developed retinal detachment due to severe PVR. Grade 0 PVR was observed in 4 (66.7%) eyes, grade 1 in 1 (16.7%) eye and grade 5 in 1 (16.7%) eye. The difference in proportion of eyes with severe PVR was significantly lower in the treatment group compared to controls at day 28 (16.7% vs 83.3%, p=0.02). Mean levels of IL-6, CRP, PDGF-BB and PLGF were similar between the 2 groups at day 28, and between baseline and day 28 within each group.

Conclusion: Liposomes as a drug delivery system for steroids could be an effective adjunctive treatment for patients with retinal detachment at high risk of developing PVR.

Introduction

Proliferative vitreoretinopathy (PVR) is a blinding condition that can occur secondary to penetrating ocular trauma or rhegmatogenous retinal detachment (RRD). This process can result in massive tractional detachment of the retina and intraretinal scarring leading to loss of vision.[1] The anatomical success rates of surgery for RRD complicated by PVR is only 69-75% compared to 98% in RRD without PVR.[2, 3] When PVR is present, multiple surgeries are often required to reattach the retina, frequently with unsatisfactory visual outcomes.[3] In addition, patients with PVR incur twice the amount of healthcare resources compared to patients without PVR.[3]

Adjunctive pharmacological treatment for PVR have shown promising results in animal models of PVR but have so far yielded less satisfactory outcomes in clinical trials.[4-12] Inflammation is a well-established step in PVR pathogenesis.[13, 14] Thus, steroids, with their anti-inflammatory properties, would appear to be an ideal solution for the treatment of PVR, yet clinical results have not been satisfactory. The exact reasons for the lack of efficacy are not well understood, but could be related to a lack of specificity for the inflammatory cell types in PVR, insufficient drug concentration to reach therapeutic thresholds, inability to maintain therapeutic levels for a sustained duration, suboptimal timing of treatment and patient selection. Some of these problems can be circumvented by the use of a targeted and sustained drug delivery system. Liposomal encapsulation of glucocorticoids have several beneficial effects, including the enhancement of therapeutic index by prolonging half-life, selective action on phagocytic inflammatory cells and avoidance of toxic effects from unintended action at non inflamed sites.[15] The goal of this study is therefore to investigate the efficacy of a small sized liposomal prednisolone phosphate preparation as targeted treatment to mitigate the severity PVR in an experimental minipig model of PVR.

Methods

The SingHealth Institute Animal Care and Use Committee (IACUC, Singhealth Approval Number 2019/SHS/1502) gave approval for this study. All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve eyes of 6 Gottingen minipigs were used for this study. Age range was 1 year ± 3months and weight range was 15-25kg. The minipigs were anesthetized with intraperitoneal injection of ketamine hydrochloride (35-50mg/kg) and Xylazil (5- 10mg/kg) prior to induction of PVR. Topical anaesthesia (proparacaine hydrochloride 0.5%) was applied and pupils were dilated with tropicamide 1%.

Platelet rich plasma preparation

In both procedures, platelet rich plasma (PRP) were prepared from minipig homologous blood 30 minutes prior to surgery, according to the method of Constable *et al*.[16] Arterial blood was collected from the minipig's ear artery into citrate blood tubes (1 part 3.8% sodium citrate to 9 parts whole blood). The blood was centrifuged at 1,200 rotations per min for 10 min, and the upper third of the supernatant PRP was aspirated. Platelet counts were performed on an automated Coulter counter to achieve 600,000 platelets per cubic millimetre of. 0.1ml of PRP were injected into the vitreous cavity at the end of surgery.

Induction of PVR

PVR was induced based on the method previously described by our group. Briefly, three-port valved 25-gauge pars plana vitrectomy (Constellation; Alcon) was performed, 3mm from the limbus, taking care to avoid traumatising the lens. First, posterior vitreous detachment was induced by suction, followed by core vitrectomy and shaving of the peripheral vitreous. Then, RPE detachment was induced in the inferior retina by injecting balanced salt solution (Alcon Laboratories, Fort Worth, TX) into the sub-RPE space, using a 39-gauge angled cannula. A retinotomy of 3-disc diameters was created within this area of RPE detachment with the vitrector. A 39-gauge angled cannula was then advanced into the

sub-RPE space to scrape the RPE and dislodge these cells into the vitreous cavity. A 90% fluid air exchange was performed to disrupt any remaining vitreous and to allow RPE cells to settle onto the retinal surface. Finally, 0.1 mL of PRP was injected into the vitreous cavity and all right eyes received 0.1ml of 5mg/ml liposomal prednisolone phosphate, while left eyes served as controls. Topical Tobramycin was administered 4 times a day for 5 days after induction of PVR.

Investigations and examination

The retina was examined with an indirect ophthalmoscope through a +20 D fundus lens on days 1, 7, 14, 21 and 28 by two double-masked ophthalmologists. We used a modified version of the Silicon Study Classification System, a classification of human PVR, that was also employed by Umazume's group[7] in their description of a swine model of PVR:

Grade 0: Normal retina, retinal or vitreous pigment clumps

Grade 1: Inner retinal wrinkling

Grade 2: Retinal detachment, 1 quadrant (1-3 clock hours)

Grade 3: Retinal detachment, 2 quadrants (4-6 clock hours)

Grade 4: Retinal detachment, 3 quadrants (7-9 clock hours)

Grade 5: Retinal detachment, 4 quadrants (10-12 clock hours)

Severe PVR was defined as retinal re-detachment i.e. grade 2-5. Fundus photographs and optical coherence tomography (OCT) was performed using a spectral domain (SD)-OCT system (Spectralis, Heidelberg Engineering Inc. Germany). At the end of study, minipigs were euthanized with intraperitoneal pentobarbitone (60- 150mg/kg) and study eyes were enucleated.

Collection of vitreous samples and analysis

Vitreous humor samples of at least 0.2ml were obtained at at the start of the vitrectomy procedure with the vitrector attached to a 3 ml syringe. On day 28, eyes were enucleated and the vitreous

obtained prior to paraffin fixation of the eye. Vitreous samples were stored at -80 degrees Celsius prior to analysis. The vitreous concentrations of interleukin 6 (IL-6), C reactive protein; (CRP), platelet derived growth factor BB (PDGF-BB), placental growth factor (PLGF) and vascular endothelial growth factor isoform A (VEGF-A) were determined using the Human multiplex ELISA kit from AYOXXA

Histopathology and Immunohistochemistry

Whole mini pig eye (28 days, n=12) was enucleated and fixed in a mixture of 10% neutral buffered formalin solution (Leica Surgipath , Leica Biosystems Richmond, Inc.) for 48 hours. The whole eye were then dissected to anterior and posterior segment prior to dehydration in increasing concentration of ethanol, clearance in xylene, and embedding in paraffin (Leica-Surgipath, Leica Biosystems Richmond, Inc.) Four-micron sections were cut with a rotary microtome (RM2255, Leica Biosystems Nussloch GmbH, Germany) and collected on POLYSINE^{TM} microscope glass slides (Gerhard Menzel, Thermo Fisher Scientific, Newington, CT). The sections were dried in an oven of 37^{o}C for at least 24 hour. To prepare the sections for histopathological and immunofluorescence examination, the sections were heated on a 60^{o}C heat plate, deparaffinized in xylene and rehydrated in decreasing concentration of ethanol. A standard procedure for Hematoxylin and Eosin (H&E) was performed. A light microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides and images were captured.

In parallel, immunofluorescence staining was performed. Heat-induced antigen retrieval was performed by incubating sections in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) for 20 minutes at 95-100^{o}C. The sections were then cooled down in sodium citrate buffer for 20 minutes in RT and washed three times for 5 minutes each with 1X PBS. Non-specific sites were blocked with blocking solution of 5% bovine serum albumin (BSA) in 0.1% Triton X-100 and 1XPBS for 1 hour at room temperature in a humidified chamber. The slides were then rinsed briefly with 1X PBS. A specific primary antibody shown in Table 1 was applied and incubated overnight at 4^{o}C in a humidified chamber

prepared in blocking solution. After washing twice with 1XPBS and once with 1X PBS with 0.1% tween for 10 minutes each, Alexa Fluro® 488 – conjugated fluorescein-labeled secondary antibody shown in Table 1 (Invitrogen- Molecular Probes, Eugene, OR) was applied at a concentration of 1:1000 in blocking solution and incubated for 90 minutes at RT. The slides were then washed twice with 1XPBS and once with 1X PBS with 0.1% tween for 5 minutes each, the slides were mounted on the slides with Prolong Diamond Anti-fade DAPI5 Mounting Media (Invitrogen- Molecular Probes, Eugene, OR) to visualize cell nucleic. For negative controls, primary antibody was omitted.

A fluorescence microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides and images were captured. Experiments were repeated in duplicates for the antibody.

Table 1: Primary antibodies used for immunohistochemical staining.

Antibody	Catalog No.	Company	Concentration
Alpha smooth muscle actin	710487	Thermo fisher Scientific	1:200
Glial Fibrillary Acid Protein GA5	14-9892-82	Thermo fisher Scientific	1:200
Macrophage, RAM11	M0633	DAKO	1:20
Alexa Fluor 488 goat anti−mouse IgG (H+L)	A11001	Invitrogen. Life Technologies (Invitrogen, Eugene, OR)	1:1000
Alexa Fluor 488 goat anti−rabbit IgG (H+L)	A11008	Invitrogen. Life Technologies (Invitrogen, Eugene, OR)	1:1000
Alexa Fluor 488 donkey anti−goat IgG (H+L)	A11055	Invitrogen. Life Technologies (Invitrogen, Eugene, OR)	1:1000

Statistics

Six minipigs in each group are required to achieve a power of 80% and a level of significance of 5% (two sided), for detecting a clinically significant effect size of 2 between treatment and control eyes. The Chi-square analysis (2-sided) was used for to compare proportion of eyes with severe PVR between the 2 groups. Mann Whitney U test was used to compare median PVR stage between groups. Statistical significance was set at p=0.05 and Stata version 16.0 was used for the analysis.

Results

PVR staging

On Day 7, fundal view was obscured in 5 eyes (2 in the control group and 3 in the treatment group) due to vitreous hemorrhage. By Day 14, vitreous hemorrhage had resolved, and fundal examination was possible in all eyes. On day 28, 5 eyes (83.3%) in the control group were observed to have severe PVR and detachment of the retina. Within the control group, PVR grade 1 (Figure 1) was observed in 1 (16.7%) eye, grade 2 in 1 (16.7%) eye, grade 3 in 2 (33.3%) eyes and grade 5 (Figure 1) in 2 (33.3%) eyes. Within the treatment group, only 1 (16.7%) eye developed retinal detachment due to severe PVR. Grade 0 PVR was observed in 4 (66.7%) eyes, grade 1 in 1 (16.7%) eye and grade 5 in 1 (16.7%) eye. The difference in proportion of eyes with severe PVR was significantly lower in the treatment group compared to the control group at day 28 (16.7% vs 83.3%, p=0.02). The median PVR grade was also significantly lower in the treatment group than the control group at day 28 (0 vs 3, p=0.039). Table 1 compares the PVR staging at all time points for both groups.

Table 1: Median PVR stage in treatment and control groups.

Day	Median PVR stage		p
	Treatment group (n=6)	Control group (n=6)	
0	0	0	1.0
7	0	0	0.39
14	0	1	0.11
21	0	1	0.15
28	0	3	0.039

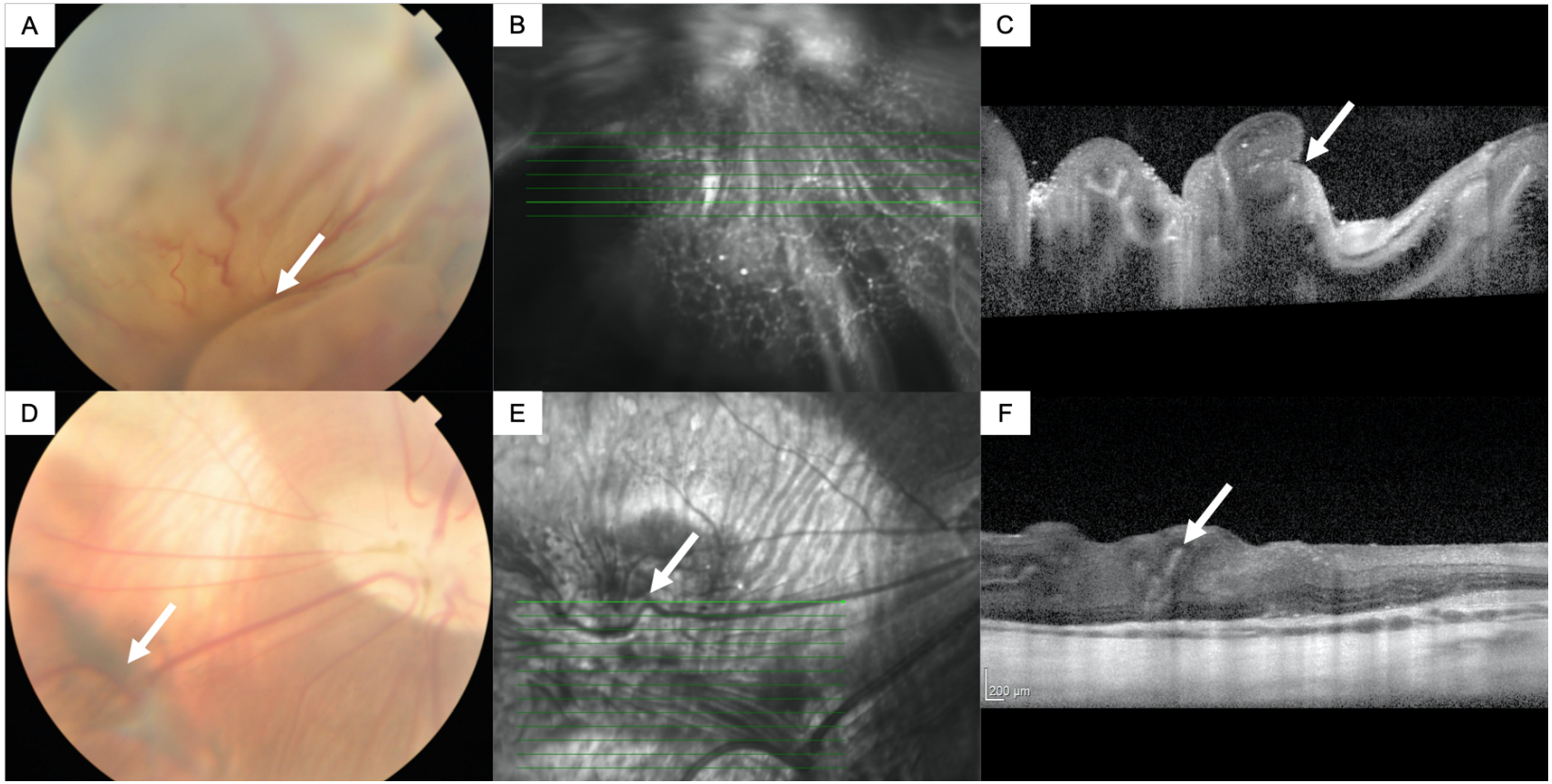

Figure 1: Fundus color photographs optical coherence tomographic (OCT) scans of an eye with grade 5 PVR (A-C) and grade 1 PVR (D-F). (A) Fundus photograph shows a totally detached retina with folds (white arrow). (B) Infrared en face image of the detached retina. The highlighted green line indicates the scan segment shown in (C), where folding of the detached retina (white arrow) can be seen. (E) Fundus photograph shows attached retina with

epiretinal/intraretinal pucker (white arrow). Infrared en face image of distortion of a retinal vessel (white arrow) crossing the area of pucker. The highlighted green line indicates the scan segment shown in (F), where distortion of the inner retina (white arrow) can be seen.

Cytokine and growth factor levels

VEGF-A levels were higher in the control group on day 28 compared to the treatment group (895.6 ± 1239.4 vs 433.0 ± 204.5, p=0.29), but this difference did not reach statistical significance. Mean levels of IL-6, CRP, PDGF-BB and PLGF were similar between the 2 groups at day 28, and between baseline and day 28 within each group (**Table 1**).

Table 2: A comparison of cytokine and growth factor levels between treatment and control groups.

Cytokine (pg/ml)	Treatment group			Control group			P**	
	Day 0	Day 28	P*	Day 0	Day 28	P*	Day 0	Day 28
IL-6	3.6 ± 0.8	3.9± 1.1	0.90	3.4 ± 1.8	3.4 ± 0.9	0.59	0.82	0.54
CRP	68.4± 24.3	72.5 ± 22.6	0.91	58.4 ± 36.1	50.1 ± 20.4	0.56	0.64	0.14
PDGF-BB	11.7 ± 6.5	19.9 ± 14.3	0.12	12.5 ± 7.6	20.0 ± 16.3	0.25	0.81	0.99
VEGF-A	318.0 ± 95.5	174.8 ± 68.9	0.10	433.0 ± 204.5	895.6 ± 1239.4	0.40	0.24	0.29
PLGF	14.2 ± 3.6	22.8 ± 30.3	0.50	18.5 ± 4.6	18.9 ± 20.1	0.96	0.11	0.80

*paired t test comparing levels of each cytokine at baseline and at day 28

** independent t test comparing levels of cytokines between treatment and control groups.

Abbreviations: IL-6, interleukin 6; CRP, C reactive protein; PDGF-BB, platelet derived growth factor BB isoform; VEGF-A, vascular endothelial growth factor isoform A; PLGF, placental growth factor.

Adverse events

Cataract and elevated intraocular pressure (IOP) are well known complications of intravitreal steroid treatment. At the end of the study, none of the treated eyes had developed cataracts. Mean intraocular pressure was 16± 3.8mmHg and 15± 4.2mmHg (p=0.89) in the treatment and control groups respectively. No spike in IOP above 21mmHg was observed throughout the duration of study.

Histology and immunohistochemistry

H&E staining demonstrated folding of the retina, a characteristic of PVR (Figure 2A). Positive staining for the following can be seen associated with the retinal folds: (2B) Macrophages, a major inflammatory cell type involved in the PVR process, (2C) Alpha smooth muscle actin, a marker for myofibroblasts derived mainly from de-differentiated RPE cells, and (2D) Glial fibrillary acid protein (GFAP), a marker of glial cells.

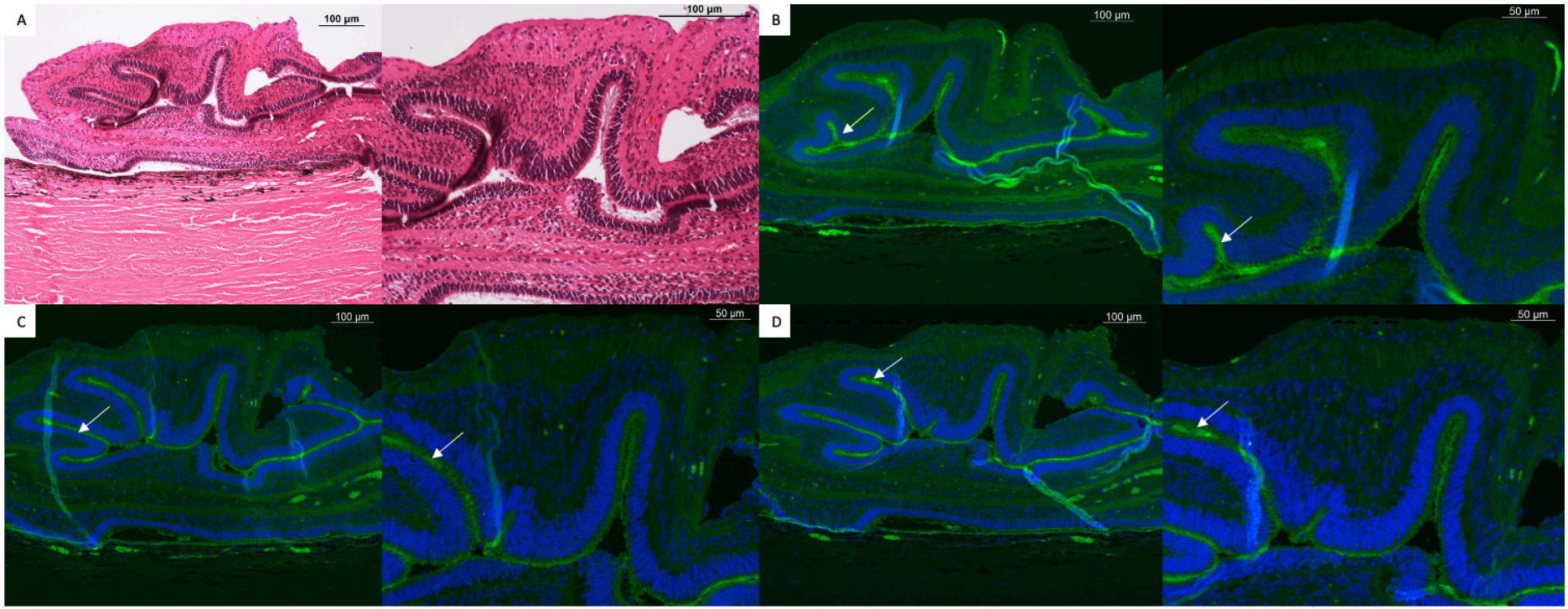

Figure 2: Histology and immunohistochemistry slides of a minipig eye with severe proliferative vitreoretinopathy (PVR) shown at 10x (left) and 20x (right) magnification. (A) H&E staining demonstrating folding of the retina, a characteristic of PVR. Positive staining for the following (white arrows) can be seen associated with the subretinal surface of these retinal folds: (2B) Macrophages, a major inflammatory cell type involved in the PVR process, (2C) Alpha smooth muscle actin, a marker for myofibroblasts derived mainly from de-differentiated RPE cells, and (2D) Glial fibrillary acid protein (GFAP), a marker of glial cells.

Discussion

This is the first study evaluating the effectiveness of liposomes as a drug delivery system for steroids to treat PVR. Eyes that received liposomal prednisolone phosphate were 5 times less likely to develop retinal re-detachment from severe PVR and had significantly lower median PVR staging at 4 weeks after PVR induction. Intravitreal cytokine and growth factor levels similar in both groups at day 28, except for VEGF-A which was higher in the control group but did not reach statistical significance. In the following sections, we discuss the role of inflammation in PVR, the evidence for steroids as an adjunctive treatment of PVR, why it has not worked in the past, and provide a hypothesis for the efficacy of liposomal prednisolone phosphate observed in our study.

Following retinal detachment, a cascade of cellular events occurs to facilitate retinal repair. Inflammation is believed to be a key driver of this process. In particular, clinical risk factors of PVR suggest that when inflammation exceeds an as yet unknown threshold in eyes with retinal detachment, the balance is tipped from retinal repair to PVR.[13] Briefly, these sequences of events begin with a break down in the blood retinal layer, exposing the immunoprivileged intraocular environment to macrophages. Macrophages not only release pro-inflammatory elements, but also promote photoreceptor cell death by apoptosis through monocyte chemoattractant protein-1 (MCP-1) At the same time, sustained separation of the neurosensory retina leads to ischemia and neuronal cell death, and glial elements including microglia and muller cells promote retinal remodeling to replace dying photoreceptors that eventually leads to retinal shortening (intraretinal PVR). Muller cells extend their processes into the subretinal space where they interact with macrophages, microglia and RPE cells to form subretinal membranes (subretinal PVR). RPE cells detach from the bruch's membrane and migrate through retinal breaks into the vitreous cavity, where they undergo epithelial mesenchymal transition (EMT). In this process, EMT inducing transcription factors induce the expression of genes that maintain the mesenchymal state and suppress the epithelial state. Cell surface epithelial cadherin

(E-cadherin) molecules on RPE cells which normally maintain the structural integrity of the RPE monolayer are suppressed, and RPE cells gain a spindle shaped morphology and express markers associated with mesenchymal cells, such as neural cadherin (N-cadherin).[13] A detailed discussion of the various pathways of EMT induction is beyond the scope of this discussion, but relevant to inflammation is the role of macrophages. In lung carcinoma cells, macrophages secrete Il-6 which is responsible for inducing EMT by activating the cyclooxygenase 2 (COX2), prostaglandin E2 (PGE2) and beta catenin signaling pathways. Subsequently, EMT-induced immunosuppressive effects have been observed which includes, in part, recruitment of M2 macrophages that secrete immunosuppressive, angiogenic and chemotactic factors that further enhance the survival, proliferation and migration of the transformed mesenchymal cells.[18] Whether M2 macrophage recruitment occurs in PVR require further study but may explain why anti-inflammatory treatment after EMT has already occurred (i.e. in PVR grade C or worse) is less ineffective, as discussed in the following section.

The potential of steroid treatment for PVR is has been well investigated, with a number studies conducted evaluating triamcinolone acetonide (TA) or dexamethasone, given intravitreally, for improving anatomical outcomes in patients with PVR with generally small effect sizes.[5, 6, 8-12] Table 3 summarises these studies. In a randomised controlled trial (n=75), Ahmadieh et al injected intravitreal TA 4mg in eyes with PVR grade C or worse and found a single surgery success rate of 84.2% compared to 78.4% in controls at 6 months (p=0.5).[9] Acar et al conducted a case control study (n=72) in which eyes with PVR grade C or worse received adjunctive intravitreal TA 4mg during surgery (group 1) or did not receive any steroid (group 2). Re-detachment rates were marginally lower (12.5%) in group 1 compared to group 2 (21.87%, P = 0.349).[8] These studies show that intravitreal TA in eyes with PVR grade C appeared to have a small but statistically insignificant benefit in terms of anatomical outcomes. A more potent steroid may produce a greater clinical effect. Dexamethasone has 5 times greater potency than TA and may achieve higher vitreous concentrations due to its higher hydrophilicity but suffers from short half-life thus necessitating a sustained release drug delivery system.[5] In a more recent study, the efficacy of a slow release dexamethasone (700ug) implant

(Ozurdex) injected intravitreally in eyes with PVR grade C was investigated in a randomised controlled trial enrolling 140 patients. Again, anatomic success at 6 months did not differ between the intervention and control groups (49.3% vs. 46.3%, p=0.73). However, Ozurdex significantly reduced the incidence of cystoid macular edema in treated eyes vs controls (42.7% vs 67.2%, p=0.004), thus demonstrating some anatomical benefit in these eyes.[5]

Table 3: Summary of studies of steroids as adjunctive therapy for proliferative vitreoretinopathy.

Author, year	Sample size	Study design	Inclusion criteria	Route of administration	Steroid	Results
Acar et al, 2010[8]	72	Case control	PVR grade C1 or worse	Intravitreal	TA 4mg	Redetachment rates were 12.50% in treatment group and 21.87% in controls (P = 0.349).
Ahmadieh et al, 2008[9]	75	RCT	PVR grade C or worse	Intravitreal	TA 4mg	Retinal reattachment without any reoperation in 32 eyes 84.2% and 78.4% in the adjunctive treatment and control groups, respectively, at 6 months (P = 0.5).
Balie E et al, 2010[10]	34	RCT	RRD	Pre op subconjunctival	Dexamethasone diphosphate 10mg	Statistically significant decrease in laser flare measurements at the 1-week postoperative visit.
Banerjee et al 2017[5]	140	RCT	PVR grade C	Intravitreal	Ozurdex (slow release dexamethasone implant) at vitrectomy and at silicone oil removal	Anatomical success was similar between the 2 groups (49.3% vs. 46.3%, adjunct vs. control; odds ratio, 0.89; 95% confidence interval, 0.46-1.74; P = 0.733).
Cheema et al, 2007[6]	24	Prospective, non-comparative	PVR grade C	Intravitreal	TA 4 mg	87.5% of patients had anatomical success at the final follow-up.
Chen et al, 2011[11]	37	Retrospective case series	PVR grade C	Intravitreal	TA 2mg at vitrectomy and 2mg at silicone oil removal	Retina was reattached in 36 (97.3%) eyes at the last visit.
Dehghan et al 2010[12]	52	RCT	RRD	Oral	Prednisolone 1mg/kg for 10 days	Postoperative PVR in the treatment and placebo groups 1 versus 3 (p=0.33)

Abbreviations: RCT, randomised controlled trial; PVR, proliferative vitreoretinopathy; RRD, rhegmatogenous retinal detachment; TA, triamcinolone acetonide

A possible reason for the small effect size in these studies could be that inflammation is the predominant event in early PVR but plays a relatively minor role in later stages where cellular proliferation and EMT become the major processes. A trial conducted by Asaria et al in 2001 provided some basis for this hypothesis.[19] This was a randomised placebo controlled clinical trial involving 174 participants with rhegmatogenous retinal detachment viewed to be at high risk of developing postoperative PVR (i.e. pre-PVR patients). The treatment group was given a continuous intraocular low molecular weight heparin (LMWH) and 5 fluorouracil (5-FU) infusion during surgery to repair the retinal detachment. The incidence of postoperative PVR was significantly lower in the treatment group than the placebo group (12.6% vs 26.4%, p=0.02). LMWH exerts anti-inflammatory action via inhibition of pro-inflammatory cytokines while 5-FU inhibits fibroblast proliferation, thus targeting different stages of the PVR process. A subsequent trial in 2007 by Wickham et al explored the use of LMWH+5FU in an unselected sample of 641 patients with rhegmatogenous retinal detachments found no significant differences in postoperative PVR rates (4.9% vs 7.0%, in treatment and placebo groups respectively, p=0.309) but patients in the treatment group who had non macular involving detachment had significantly worse visual acuity, presumably related to 5-FU toxicity.[20] Taking these 2 studies together, the choice of a pre-PVR study population may be an important factor in achieving a greater effect size and minimizing the risk of visual loss with this treatment.

There is also evidence from animal studies for the effectiveness of steroid, given in the inflammatory phase of PVR. In a macrophage induced rabbit model of PVR, a combination treatment of TA given immediately after PVR induction, followed by daunomycin given on day 6, was most effective in preventing retinal re-detachment from PVR (8.3%) compared to TA alone (33.3%), daunomycin alone (16.1%) and controls (83.3%).[21] In another study, using the same PVR model, TA, liposomal daunomycin, free daunomycin and empty liposomes were injected at the point of PVR induction. PVR with retinal detachment occurred in 13.3%, 33.3%, 50% and 77.5% respectively.[22] In addition to demonstrating the effectiveness of TA given early in the disease process, the study showed that encapsulation of daunomycin in liposomes increased its effectiveness in preventing PVR compared to free daunomycin.

Thus, with the observations gleaned from the abovementioned studies, we postulate that liposomal prednisolone phosphate may exert its therapeutic effect in the following ways. First, prednisolone phosphate given early in the inflammatory phase of PVR keeps inflammatory pathways in check thus preventing runaway inflammation from igniting the PVR cascade. Second, encapsulation of steroid within liposomes enhance their dwell time in the vitreous cavity, extending their therapeutic effect well beyond the inflammatory phase of PVR. Finally, liposome encapsulation of prednisolone phosphate alters their site of action from T cells to predominantly macrophages, the major inflammatory cell type in PVR, and shifts their phenotype from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype, thereby tilting the balance back in favor of retinal repair rather than the over-exuberant scarring seen in PVR. Further studies are needed to fully explore and elaborate these mechanisms.

There are some limitations to our study. The sample size is small, but adequately powered to detect the large effect size of liposomal steroid as demonstrated in our study. As this is a pilot study with the aim of demonstrating proof of efficacy, we did not design the study to evaluate the pharmacokinetics of liposomal steroid, nor assess its retinal toxicity. These questions will be addressed in a subsequent study. Our study was not designed to assess the timing of treatment on treatment efficacy, but our group had previously shown in an experimental model of PVR that inflammatory cytokines peak within the first 2 weeks of PVR induction, well before definite signs of PVR can be observed on fundoscopic examination. It can thus be inferred that anti-inflammatory therapy should be initiated as early as possible to optimize treatment outcomes. Further studies are needed to clarify this issue. In conclusion, we demonstrated the effectiveness of a single dose of intravitreal liposomal prednisolone phosphate in mitigating the severity of PVR in a minipig model. Liposomes as a drug delivery system for steroids could be an effective adjunctive treatment for patients with retinal detachment at high risk of developing PVR. Further studies are needed to validate these findings, optimize the dose, evaluate

different routes of administration and to evaluate potential synergistic effects of liposomal steroid with anti-proliferative therapeutics on the treatment of PVR.

References

1. Newsome DA, Rodrigues MM, Machemer R. Human massive periretinal proliferation. In vitro characteristics of cellular components. *Archives of ophthalmology* 1981;99:873-880.

2. Wong CW, Wong WL, Yeo IY, et al. Trends and factors related to outcomes for primary rhegmatogenous retinal detachment surgery in a large asian tertiary eye center. *Retina* 2014;34:684-692.

3. Patel NN, Bunce C, Asaria RH, Charteris DG. Resources involved in managing retinal detachment complicated by proliferative vitreoretinopathy. *Retina* 2004;24:883-887.

4. Hou H, Nudleman E, Weinreb RN. Animal Models of Proliferative Vitreoretinopathy and Their Use in Pharmaceutical Investigations. *Ophthalmic Res* 2018;60:195-204.

5. Banerjee PJ, Quartilho A, Bunce C, et al. Slow-Release Dexamethasone in Proliferative Vitreoretinopathy: A Prospective, Randomized Controlled Clinical Trial. *Ophthalmology* 2017;124:757-767.

6. Cheema RA, Peyman GA, Fang T, Jones A, Lukaris AD, Lim K. Triamcinolone acetonide as an adjuvant in the surgical treatment of retinal detachment with proliferative vitreoretinopathy. *Ophthalmic Surg Lasers Imaging* 2007;38:365-370.

7. Gagliano C, Toro MD, Avitabile T, Stella S, Uva MG. Intravitreal Steroids for the Prevention of PVR After Surgery for Retinal Detachment. *Curr Pharm Des* 2015;21:4698-4702.

8. Acar N, Kapran Z, Altan T, Unver YB, Pasaoglu E. Pars plana vitrectomy with and without triamcinolone acetonide assistance in pseudophakic retinal detachment complicated with proliferative vitreoretinopathy. *Jpn J Ophthalmol* 2010;54:331-337.

9. Ahmadieh H, Feghhi M, Tabatabaei H, Shoeibi N, Ramezani A, Mohebbi MR. Triamcinolone acetonide in silicone-filled eyes as adjunctive treatment for proliferative vitreoretinopathy: a randomized clinical trial. *Ophthalmology* 2008;115:1938-1943.

10. Bali E, Feron EJ, Peperkamp E, Veckeneer M, Mulder PG, van Meurs JC. The effect of a preoperative subconjuntival injection of dexamethasone on blood-retinal barrier breakdown following scleral buckling retinal detachment surgery: a prospective randomized placebo-controlled double blind clinical trial. *Graefes Arch Clin Exp Ophthalmol* 2010;248:957-962.

11. Chen W, Chen H, Hou P, Fok A, Hu Y, Lam DS. Midterm results of low-dose intravitreal triamcinolone as adjunctive treatment for proliferative vitreoretinopathy. *Retina* 2011;31:1137-1142.

12. Dehghan MH, Ahmadieh H, Soheilian M, et al. Effect of oral prednisolone on visual outcomes and complications after scleral buckling. *Eur J Ophthalmol* 2010;20:419-423.

13. Pastor JC, Rojas J, Pastor-Idoate S, Di Lauro S, Gonzalez-Buendia L, Delgado-Tirado S. Proliferative vitreoretinopathy: A new concept of disease pathogenesis and practical consequences. *Prog Retin Eye Res* 2016;51:125-155.

14. Wong CW, Cheung N, Ho C, Barathi V, Storm G, Wong TT. Characterisation of the inflammatory cytokine and growth factor profile in a rabbit model of proliferative vitreoretinopathy. *Sci Rep* 2019;9:15419.

15. Wong CW, Wong TT. Posterior segment drug delivery for the treatment of exudative age-related macular degeneration and diabetic macular oedema. *Br J Ophthalmol* 2019;103:1356-1360.

16. Constable IJ, Oguri M, Chesney CM, Swann DA, Colman RW. Platelet-induced vitreous membrane formation. *Invest Ophthalmol* 1973;12:680-685.

17. Hida T, Chandler DB, Sheta SM. Classification of the stages of proliferative vitreoretinopathy in a refined experimental model in the rabbit eye. *Graefes Arch Clin Exp Ophthalmol* 1987;225:303-307.

18. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. *Nat Rev Mol Cell Biol* 2019;20:69-84.

19. Asaria RH, Kon CH, Bunce C, et al. Adjuvant 5-fluorouracil and heparin prevents proliferative vitreoretinopathy : Results from a randomized, double-blind, controlled clinical trial. *Ophthalmology* 2001;108:1179-1183.

20. Wickham L, Bunce C, Wong D, McGurn D, Charteris DG. Randomized controlled trial of combined 5-Fluorouracil and low-molecular-weight heparin in the management of unselected rhegmatogenous retinal detachments undergoing primary vitrectomy. *Ophthalmology* 2007;114:698-704.

21. Hui YN, Hu D. Prevention of experimental proliferative vitreoretinopathy with daunomycin and triamcinolone based on the time course of the disease. *Graefes Arch Clin Exp Ophthalmol* 1999;237:601-605.

22. Hui YN, Liang HC, Cai YS, Kirchhof B, Heimann K. Corticosteroids and daunomycin in the prevention of experimental proliferative vitreoretinopathy induced by macrophages. *Graefes Arch Clin Exp Ophthalmol* 1993;231:109-114.

8

Liposomal drug delivery system for anti-inflammatory treatment after cataract surgery: A Phase I/II clinical trial

Chee Wai Wong[1,2,3]
Edmund Wong[1,2,3]
Josbert M. Metselaar[5]
Gert Storm[3,5]
Tina T. Wong[1,2,3]

1. 1. Singapore National Eye Centre (SNEC), 11 Third Hospital Avenue, Singapore 168751
2. Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore 168751
3. Duke-National University of Singapore Medical School, 8 College Rd, Singapore 169857
4. Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands.
5. Department of Experimental Molecular Imaging, University Clinic and Helmholtz Institute for Biomedical Engineering, RWTH Aachen University, Aachen 52074, Germany

Drug Delivery and Translational Research (under revision)

Abstract

Purpose: Liposomes as a drug delivery system may overcome the problems associated with non-compliance to eyedrops and inadequate control of inflammation after cataract surgery. We evaluated the safety and efficacy of a single subconjunctival injection of liposomal prednisolone phosphate (LPP) for the treatment of post-cataract surgery inflammation.

Methods: This is a Phase I/II, open-label non-comparative interventional trial of patients undergoing cataract surgery. All patients received a single injection of subconjunctival LPP intraoperatively. The primary outcome measure was the proportion of eyes with an anterior chamber cell count of 0 at postoperative month 1. Ocular and non-ocular adverse events, including elevated intraocular pressure, rebound iritis and pseudophakic macular edema were monitored.

Results: Five patients were enrolled in this study. The mean age was 66.6 ± 6.2 and 4 (80%) were male. The proportion of patients with AC cell grading of 0 was 0%, 80%, 80% and 100% at day 1, week 1, month 1 and month 2 after cataract surgery, respectively. Mean laser flare photometry readings were significantly elevated at week 1 after cataract surgery (48.8± 18.9, p=0.03) compared to baseline, decreasing to 25.8 ± 9.2 (p=0.04) at month 1 and returned to baseline by month 2 (10.9 ± 5.1, p=1.0). No ocular or non-ocular adverse events were observed.

Conclusions: Liposomal prednisolone phosphate, administered as a single subconjunctival injection intraoperatively, can be a safe and effective treatment for post-cataract surgery inflammation. The delivery of steroids with a liposomal drug delivery system could potentially replace eyedrops as anti-inflammatory therapy following cataract surgery.

Introduction

Cataract surgery is, by far, the most common intraocular surgical procedure performed: an estimated 4 million cataract surgeries are performed annually in the United States alone. Anterior segment inflammation is an inevitable sequela after cataract surgery and is the leading cause of pain, reduced vision, and delayed recovery after surgery. Such inflammation, if left unchecked, can lead to corneal edema, raised intraocular pressure, and pseudophakic macular edema.[1] These complications can have an irreversible negative impact on vision post-cataract surgery.[2]

Corticosteroids are the first-choice treatment for post-cataract surgery anterior segment inflammation, and the current gold standard is topical eyedrops therapy. However, several limitations exist for topical eyedrop administration: 1. The limited capacity of the conjunctival sac (25ul), limited drug residence time, and drainage of the drug through the nasolacrimal duct reduces bioavailability. Ocular absorption of topically applied drugs is typically less than 5%[3]; 2. Steroid eye drops are cloudy suspensions that can cause blurring of vision and ocular irritation; 3. Compliance with the treatment regimen is a challenge. 4. Elderly patients often face difficulty with administering eyedrops. Improper eyedrop technique can result in trauma to the cornea or even contaminate the eye and increase the risk of endophthalmitis.[4 5]

Various drug delivery systems have attempted to solve the abovementioned problems posed by eyedrops, including polymer- and lipid-based nanomaterials.[6] The most studied nanocarriers in ophthalmic disease are liposomes, which have the advantages of being biocompatible and biodegradable.[7] Pegylated liposomal formulations of water-soluble corticosteroids have shown promising outcomes in systemic inflammatory diseases such as rheumatoid arthritis (Phase I/II) and ulcerative colitis (Phase IIa).[8] Interestingly, these liposomes are efficiently taken up by macrophages in inflamed tissue, thereby increasing their tissue specificity and may increase the

therapeutic index of the encapsulated anti-inflammatory agents.[9] Besides liposomes, a few non liposomal drug delivery systems that deliver steroids for the treatment of post cataract surgery inflammation are FDA approved and commercially available. These include 1.Surodex (Oculex Pharmaceuticals Inc, Sunnyvale, California), an intraocular implant composed of a biodegradable lactic acid/glycolic acid copolymer loaded with 60ug of dexamethasone,[10] 2.Dexycu® (Icon Bioscience, Inc. Sunnyvale, CA, USA), an intracameral injection using Icon's Verisome™ (Icon Bioscience, Inc.) drug delivery technology for dexamethasone,[11 12] and 3. Dextenza (Ocular Therapeutix, Inc, Bedford, Massachusetts), an intracanalicular implant containing 0.4mg dexamethasone.[13] A common downside of these drug delivery systems is the need for invasive routes of delivery (intracameral and intracanalicular) with the potential to damage ocular structures (the cornea, iris and canaliculus).

We previously studied the efficacy of a PEG-liposomal formulation of liposomal prednisolone phosphate, administered as a single subconjunctival dose, in the treatment of experimental uveitis in rabbit eyes.[14] Rabbits that received subconjunctival liposomal prednisolone phosphate (LPP) had significantly lower mean inflammatory scores than untreated controls after induction of uveitis on Day 4 (LPP vs. controls, p=0.049) and 8 (LPP vs. controls, p=0.007), and lower scores than rabbits given topical Pred Forte 1% four times a day on Day 8 (p=0.03). Histology and immunostaining confirmed the co-localization of liposomes with macrophages in inflamed tissue, and liposomes persisted in the eye for at least one month.[14] These attributes are desirable as an elegant solution for post-cataract surgery ocular inflammation: 1. subconjunctival injections of antibiotics and free steroids are already routinely delivered after cataract surgery under anesthesia: no additional steps are required; 2. the duration of action of this liposomal steroid formulation will provide adequate coverage for post-cataract surgery inflammation, which typically lasts for 2-4 weeks.

To determine if a single subconjunctival injection of liposomal steroids can replace eyedrops for the management of post-cataract surgery inflammation, we conducted a Phase I/II clinical study with the

primary aim of evaluating safety and a secondary aim of evaluating the efficacy of the abovementioned liposomal prednisolone phosphate formulation.

Methods

Study Design

We conducted a 90-day open-label, single-arm, non-comparative study of patients with significant cataract requiring surgery, enrolled from the Singapore National Eye Centre in December 2019. The study was conducted in accordance with the Singapore Human Biomedical Research Act and with regulatory approval by the Health Sciences Authority of Singapore. The study protocol was approved by the Singhealth Centralised Institutional Review Board (protocol number R1600/99/2018), and all participants gave written informed consent.

Patient Inclusion Criteria

All patients aged 50 or older with significant cataract, with visual acuity worse than 20/40 and scheduled for phacoemulsification with posterior chamber intraocular lens implantation were eligible for this study. Only patients who have had previous cataract surgery in the fellow eye and did not have a documented steroid response (defined as steroid-related increase in intraocular pressure ≥ 28mmHg) were included in this study.

Patient Exclusion Criteria

Exclusion criteria included the following: the presence of any ocular co-morbidity other than cataract; complicated cataracts such as posterior polar cataracts and subluxated or dislocated cataracts; a history of steroid response; treatment with any form or route of ocular steroids or NSAIDs in the study eye within the past three months; requiring treatment with steroids by any route during the study; known allergy to prednisolone phosphate; poor visual acuity in the fellow eye of worse than 20/40.

Liposomal steroid preparation

Liposomes preparation has been previously described.[15] In brief, dipalmitoyl phosphatidyl choline (DPPC), cholesterol, and PEG2000 distearoyl phosphatidylethanolamine (PEG-DSPE) were added in a 62%, 33%, and 5% molar ratio. Prednisolone phosphate was dissolved in water for injection and lipids were dissolved in absolute ethanol at 65 °C. The alcoholic lipid solution was mixed with the aqueous steroid solution at 65 °C, forming a multilamellar vesicle dispersion. This desired particle size of approximately 100 nm in diameter was obtained by repeated homogenization cycles using an Avestin C55 high-shear homogenizer (Avestin, Mannheim, Germany). Ultrafiltration using membranes with a molecular weight cut off of 30 kDa was performed to remove unencapsulated prednisolone phosphate and replaced with clean dispersion buffer. Lastly, sterile filtration of the lipid dispersion was performed, collected in vials and stored between 2 and 8 °C. The characteristics (Table 1) and drug release profile of this formulation in aqueous medium and plasma has previously been published. In such media, they show good drug retention properties, which is essential to ensure transport and delivery of the liposomal encapsulated drug at the target cells (e.g. macrophages) in the inflamed site.[8 9 16] The formulation studied here is the same as the formulation developed and evaluated by Lobatto et al. With this formulation, neither in vitro (buffer, 37 °C) nor in vivo (in the blood circulation) release of encapsulated drug from the liposomes was observed.[17]

Table 1: Characteristics of liposomes

	Empty liposomes (C)	Prednisolone liposomes (LPP)
Size (nm)	120 ± 5	110 ± 6
Polydispersity index	0.014	0.040
Zeta potential (meV)	-0.6	+4.3
Drug concentration	-	5mg/ml
Encapsulation Efficiency (EE%)	-	10%

Study Procedures

Following successful screening, all eligible patients were allocated to treatment with liposomal prednisolone phosphate on the day of phacoemulsification cataract surgery. Cataract surgery was performed by a single surgeon (Chee Wai Wong). At the end of routine phacoemulsification and posterior chamber intraocular lens implantation, the surgeon injected a volume of 100μL of liposomal prednisolone phosphate (5 mg/ml) into the superior bulbar conjunctiva using a 30-gauge needle on an insulin syringe under operating microscope guidance (Figure 1).

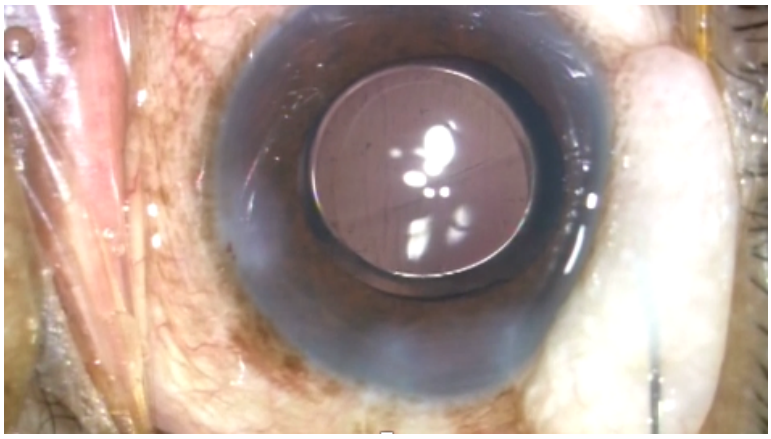

Figure 1: Subconjunctival injection of liposomal prednisolone phosphate at the end of cataract surgery.

Study visits were scheduled at postoperative day 1, week 1, month 1, and month 2. We performed the following measurements at each study visit: subjective pain score (on a scale of 1-10 with 10 being the most painful), logMAR best-corrected visual acuity, laser flare photometry, slit lamp examination, measurement of intraocular pressure, and dilated fundoscopy.

Visual acuity was measured with the logarithm of the minimal angle of resolution (logMAR) chart (Lighthouse International, New York, New York, USA) at 4 meters, with subjects wearing their current optical correction (glasses or contact lenses). If the largest number could not be identified at 4 meters, the chart was brought closer to the subject, then counting fingers, hand motion, or light perception vision was assessed.

We performed a standardized ophthalmic examination, including intraocular pressure (IOP) measurement with the Goldman applanation tonometry (Haag-Streit, Konig, Switzerland). After pupil dilation, the posterior segment was evaluated with a 78-diopter (D) lens at 16X magnification, and a 20D lens was used to examine the peripheral fundus. The severity of anterior chamber inflammation was graded based on the number of anterior chamber cells using a 1x1mm slit lamp field size and anterior chamber flare with the Standardization of Uveitis Nomenclature (SUN) criteria.[18]

Anterior chamber flare was quantified at each visit using laser flare photometry (LFP) with the Kowa FM 700 laser flare meter (Kowa Company Ltd, Nagoya, Japan). Optical coherence tomography (OCT, Spectralis, Heidelberg Engineering, Heidelberg, Germany) was performed at the screening visit, postoperative months 1, 2, and 3. OCT scans were qualitatively assessed for the presence of pseudophakic macular edema.

Outcome Measures

The primary outcome measure was the proportion of eyes with an anterior chamber cell count of 0 at postoperative month 1. Secondary endpoints include the proportion of patients with subjective pain score of zero at each visit, the proportion of patients with anterior chamber flare grading of 0 at each visit, mean LFP readings at each visit, and the proportion of patients with cystoid macular edema at any postoperative visit.

Safety Outcomes

We examined the injection site at each visit for any localized redness, swelling, or other signs of infection. Patients with rebound iritis, defined as a one-step increase in severity in anterior chamber cells or flare based on the SUN criteria, at any visit will be started on topical pred forte 1% eyedrops three hourly and tapered at the treating physician's discretion. Elevation of IOP more than 21mmHg was monitored and treated with IOP lowering eyedrops if required. All adverse events, whether informed by the patient or detected by study investigators, were recorded in the case report forms.

Statistical Analysis

STATA SE version 13.0 was used for the analysis. Descriptive analysis of the primary and secondary endpoints was performed. Proportions were presented as percentages and means with standard deviation. Medians were analysed with the paired samples Wilcoxon test to compare baseline values with other time points. Analysis of flare meter readings was performed with the paired t-test, comparing values at various time points with baseline readings. Multiple comparisons were adjusted with the Bonferroni correction. All tests are 2 sided with significance at $p<0.05$.

Results

Patient Demographics

Five patients were enrolled in this study. The mean age was 66.6 ± 6.2, 4 (80%) are male, and the ethnic distribution was 2 (40%) Chinese, 2 (40%) Malay and 1 Indian (20%). Four (80%) study eyes were right eyes. Four patients had underlying type 2 diabetes mellitus without diabetic retinopathy or maculopathy. Four patients had nuclear sclerotic cataracts of medium density, and one patient had a dense brunescent cataract. The mean baseline IOP was 18.2 ± 1.8mmHg, and mean baseline LFP readings were 8.8 ± 5.1 ph/ms and 10.5 ± 4.0 ph/ms for the study eye and fellow eye, respectively. All eyes had AC cell and flare grading of 0 at baseline.

Outcomes

The proportion of patients with AC cell grading of 0 was 0%, 80%, 80% and 100% at day 1, week 1, month 1 and month 2 after cataract surgery, respectively. Similarly, AC flare clearance was seen in 0%, 100%, 80% and 100% at day 1, week 1, month 1 and month 2 after cataract surgery, respectively (Figure 2). None of the patients complained of pain during the injection or at any time point after surgery, except for 1 patient who complained of a pain score of 4/10 1 day after surgery. He described this pain as similar to the pain that he had after cataract surgery in the fellow eye. The pain

decreased to a score of 2 by week 1 and was entirely resolved by month 1. Baseline logmar BCVA was 0.65±0.19 (20/80), improving to 0.25±0.13 (20/32) at two months postoperatively (p=0.01).

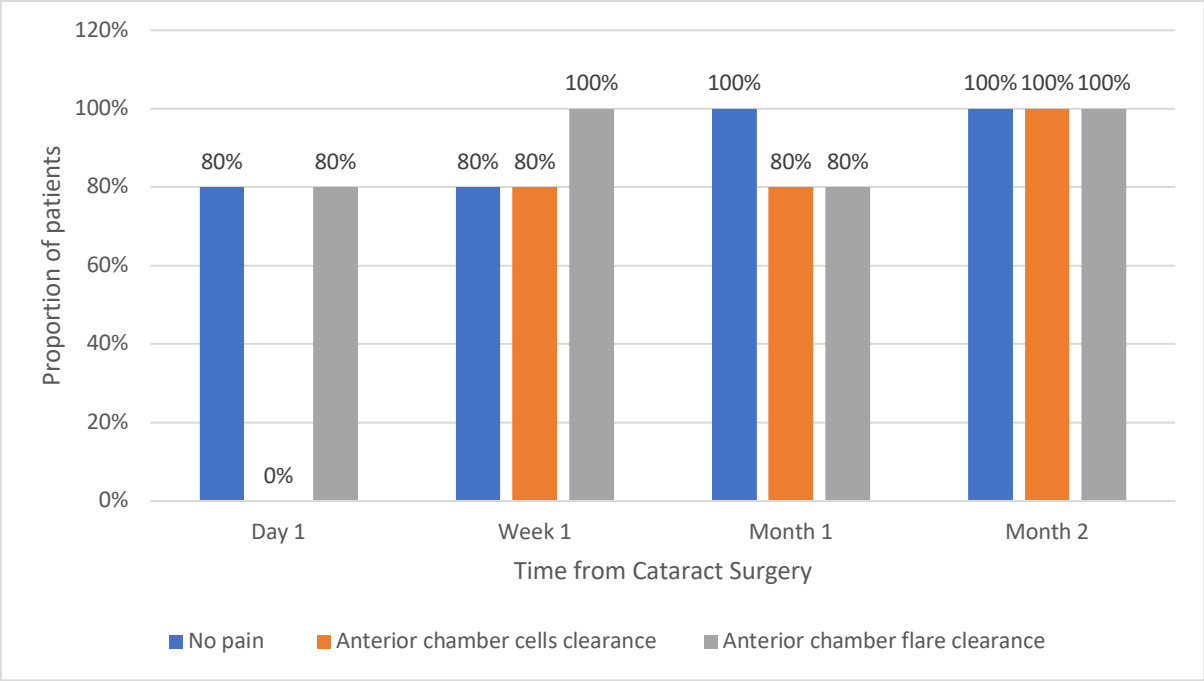

Figure 2: Proportion of patients achieving primary and secondary outcomes at various time points after cataract surgery.

Table 2: Median anterior chamber cell and flare grade, mean laser flare photometry readings and intraocular pressure at various time points, compared to baseline.

	Baseline	Day 1	P*	Week 1	P*	Month 1	P*	Month2	P*
Median anterior chamber cell grade	0	0.5	0.25	0	1.0	0	1.0	0	1.0
Median anterior	0	0	1.0	0	1.0	0	1.0	0	1.0

chamber flare grade									
Mean laser Flare photometry reading, ph/ms	8.8±5.1	38.7±19.9	0.43	48.8± 18.9	0.03	25.8 ± 9.2	0.04	10.9 ± 5.1	1.0
IOP, mmHg	18.2 ± 1.9	15.2 ± 1.5	0.04	16.6 ± 1.5	1.0	12.6 ± 2.3	0.005	12.6 ± 3.0	0.03

Abbreviations: IOP, intraocular pressure.

* Bonferroni adjusted p values comparing values at week 1, month 1 and month 2 with baseline values.

Table 2 summarizes the median anterior chamber cell and flare grade, mean laser flare photometry readings and intraocular pressure at various time points, compared to baseline.

The median AC cell grading was 0.5 and the median AC flare grading was 0 on day 1 after cataract surgery. These remained at 0 at all other time points. Mean LFP readings were significantly elevated at week 1 after cataract surgery (48.8± 18.9ph/ms, p=0.03) compared to baseline, decreasing to 25.8 ± 9.2ph/ms (p=0.04) at 1 month after surgery. By Month 2, the mean LFP reading had returned to baseline (10.9 ± 5.1ph/ms, p=1.0)

Safety

No local adverse effects were noted at the injection site at all time points. None of the patients experienced rebound iritis or required additional anti-inflammatory treatment at any time after surgery. IOP remained within normal limits for all patients within the study period. None of the patients developed pseudophakic macular edema at any time within the study period.

Discussion

We conducted a phase I/II non-comparative interventional trial designed to evaluate the safety and efficacy of a novel small-sized PEGylated liposome drug delivery system for prednisolone phosphate to treat anterior chamber inflammation related to cataract surgery. We found that a single subconjunctival injection of liposomal prednisolone phosphate was well tolerated, both during the injection and postoperatively. No ocular or non-ocular adverse events were observed throughout the study period. All patients had resolution of pain, and 80% achieved complete resolution of anterior chamber cellular activity at 1 month after surgery. We did not observe rebound iritis, elevated IOP, or pseudophakic macular edema in any patient throughout the study period.

Cataract surgery involves the creation of a cornea wound, application of ultrasound energy, mechanical manipulation in the anterior chamber, and implantation of an intraocular lens. These manoeuvres induce trauma in the anterior segment of the eye and disrupts the blood aqueous barrier, invariably leading to post-operative inflammation. The need for post-operative anti-inflammatory treatment in the modern era of phacoemulsification cataract surgery was demonstrated by Lorenz et al in a multi-center randomized double-masked vehicle controlled, parallel group study. The study compared prednisolone acetate eyedrops with vehicle eyedrops, give 4 times a day for 2 weeks, and concluded that anti-inflammatory treatment should be initiated in the early postoperative period even in uneventful cataract surgery.[19] Current management of post-cataract surgery inflammation in the form of eyedrops has the disadvantages of low patient compliance and low bioavailability, leading to postoperative complications. These include rebound iritis after stopping steroid eyedrops and pseudophakic macular edema.[1] Rebound inflammation, particularly in patients with a history of uveitis or in patients non-compliant to steroid eyedrop treatment after surgery, can occur in up to 51% of patients.[20] Patients develop pain and decreased vision leading to additional clinic visits and prescriptions for topical steroids. Pseudophakic macular edema can develop in up to 22% of patients following cataract surgery.[21] The estimated cost of treatment was 5.7 times (USD$1041 vs. $5950)

higher on average in patients with pseudophakic macular edema than those without.[22] The underlying etiology could be at least in part related to inadequately controlled inflammation, particularly in patients at risk of excessive inflammation such as patients with diabetes.

Drug delivery systems to replace steroid eyedrops post-cataract surgery have been explored, with a few products now FDA approved and commercially available. Surodex (Oculex Pharmaceuticals Inc, Sunnyvale, California) is an intraocular implant composed of a biodegradable lactic acid/glycolic acid copolymer loaded with 60ug of dexamethasone.[10] In a phase II randomized clinical trial (n=60)[23], Surodex was inserted into the anterior chamber via a corneal wound at the end of extracapsular cataract extraction and compared with controls who received dexamethasone 0.1% eyedrops 4 times a day for 4 weeks. The Surodex group had significantly reduced anterior chamber inflammation from day 4 postoperatively. At 3 months post-surgery, the Surodex group had achieved flare reduction to preoperative levels but not in the control group. Therapeutic failure, defined as a need for augmentation of steroids, was seen in 5 (17.9%) eyes in the control group and 1 (3.1%) in the Surodex group. Lastly, the safety profile of Surodex was acceptable with no eyes developing glaucoma or significant endothelial cell loss at 1-year post-surgery. Ozurdex (Allergan, Irvine, CA) is a similar dexamethasone loaded implant that is commercially available and indicated for treatment of diabetic macular edema, a condition in which diabetes mellitus related damage to vascular endothelium results in intraretinal fluid accumulation and lipid exudation in the macula.[24] Dexycu® (Icon Bioscience, Inc. Sunnyvale, CA, USA) is a single intracameral injection using Icon's Verisome™ (Icon Bioscience, Inc.) drug delivery technology for dexamethasone. The Verisome™ technology is a proprietary drug delivery system that allows formulation of dexamethasone into a viscous gel. When injected into the eye, the gel coalesces to form a spherule that releases dexamethasone over time. A randomized, double-masked multicentre trial (n=394) comparing 2 different dosages of Dexycu with placebo showed that at postoperative day 8, clearance of anterior chamber cells was achieved in 25.0%, 63.1% and 66.0% of eyes in the placebo, 342ug, and 517µg treatment groups, respectively ($P < 0.001$). The safety profile of both Dexycu doses was similar to placebo, and no serious ocular adverse events were reported.[11 12] Dexycu was approved by the

FDA in 2018 and is now available commercially in the United States. Finally, Dextenza (Ocular Therapeutix, Inc, Bedford, Massachusetts) is an intracanalicular implant containing 0.4mg dexamethasone approved by the FDA for the treatment of postoperative ocular pain with a single treatment for up to 30 days.[13] In a multicentred randomized double-masked placebo-controlled phase 3 trial (n=218), significantly more patients had resolution of anterior chamber cells at postoperative day 14 in the Dextenza arm compared with placebo (52.3% versus 31.1%; $P < .0001$).[25] Although effective, there may be some drawbacks to the aforementioned drug delivery systems. Intracanalicular implants can increase tearing, and the implants can be dislodged. They may also cause discomfort and trauma to the canaliculus.[25] Intracameral injections [26 27] carry a risk of damage to intraocular structures such as the corneal endothelium and iris, and suspensions may cause transient blurring of vision.[11] In comparison, LPP is injected into the subconjunctival space thus avoiding damage to anterior segment structures, will not cause cloudy vision, cannot be dislodged, and is well tolerated.

In this study, we administered 0.1ml of 5mg/ml of liposomal prednisolone phosphate as a subconjunctival injection. This is equivalent to 0.5mg of prednisolone phosphate. In comparison, Pred Forte 1% eyedrops given 6 times a day over 4 weeks is equivalent to a total dose of 0.42mg, not taking into account the low bioavailability of eyedrops of less than 5%. In addition, the sclera can act as a depot allowing sustained release of LPP to the inflamed intraocular tissue.[14] The drug release profile of this formulation in aqueous medium and plasma has previously been published.[16] In such media, the liposomes show 100% retention of the incorporated drug, which is essential to ensure transport to and delivery of the liposome encapsulated drug to the target cells (e.g. macrophages) in the inflamed site. With this formulation, neither in vitro (buffer, 37 °C) nor in vivo (in the blood circulation) release of encapsulated drug from the liposomes was observed. This is a desired property as drug release should only take place in the target cells after uptake to achieve maximum efficacy.

Laser flare photometry is a reproducible and objective method for quantifying anterior chamber inflammation.[28] This is particularly useful for measuring the usually mild inflammation after

cataract surgery, because clinical grading systems were based on uveitis studies where the degree of inflammation is much more severe. Thus, laser flare photometry may detect more subtle inflammation that cannot be detected on slit lamp examination. This subclinical inflammation may explain the occurrence of pseudophakic cystoid macular edema several weeks after cataract surgery, despite the appearance of an apparently quiet eye.[29] In this study, flare meter readings peaked at 1 week after cataract surgery before decreasing back to baseline levels at month 2. Although clinical signs of inflammation had resolved as early as week 1 in most patients, flare meter readings suggest that low-grade subclinical inflammation resolved sometime between months 1 and 2. Despite the persistence of mild subclinical inflammation, none of our patients developed pseudophakic macular edema during the study. The persistence of subclinical inflammation could be explained by the fact that 4 out of 5 of our study patients were diabetic, which is consistent with past observations of increased disruption of the blood-aqueous barrier in diabetic patients compared with non-diabetic patients.[30] One of our study patients had a dense brunescent cataract which required higher phacoemulsification energy and more intraocular manipulation to remove, which may also explain the greater postoperative inflammation. Whether there is a need to treat this subclinical inflammation, in the absence of any subjective or objective detriment, remains debatable. These issues should be addressed in a larger Phase II study to explore higher doses of liposomal prednisolone phosphate in patients at greater risk of increased post-cataract surgery inflammation.

A strength of this study is the combination of both objective (laser flare photometry and OCT) and subjective measures of efficacy. Major limitations of this study include the small number of patients and a lack of a comparison group with the current standard of care. Although the treatment outcomes are consistent in all 5 patients, a much larger sample size and a comparison group with steroid eyedrops are needed to validate the results of our study. In conclusion, LPP administered as a single subconjunctival injection as a concluding step in cataract surgery could potentially replace steroid eyedrops for patients following cataract surgery. The safety and efficacy of LPP will require validation in larger clinical trials.

References

1. Liu YC, Wilkins M, Kim T, Malyugin B, Mehta JS. Cataracts. Lancet 2017;**390**(10094):600-12 doi: 10.1016/S0140-6736(17)30544-5[published Online First: Epub Date]|.

2. McColgin AZ, Heier JS. Control of intraocular inflammation associated with cataract surgery. Curr Opin Ophthalmol 2000;**11**(1):3-6

3. Urtti A, Salminen L. Minimizing systemic absorption of topically administered ophthalmic drugs. Surv Ophthalmol 1993;**37**(6):435-56

4. Schwartz GF, Hollander DA, Williams JM. Evaluation of eye drop administration technique in patients with glaucoma or ocular hypertension. Curr Med Res Opin 2013;**29**(11):1515-22 doi: 10.1185/03007995.2013.833898[published Online First: Epub Date]|.

5. Tatham AJ, Sarodia U, Gatrad F, Awan A. Eye drop instillation technique in patients with glaucoma. Eye (Lond) 2013;**27**(11):1293-8 doi: 10.1038/eye.2013.187[published Online First: Epub Date]|.

6. Weng Y, Liu J, Jin S, Guo W, Liang X, Hu Z. Nanotechnology-based strategies for treatment of ocular disease. Acta Pharm Sin B 2017;**7**(3):281-91 doi: 10.1016/j.apsb.2016.09.001[published Online First: Epub Date]|.

7. Agarwal R, Iezhitsa I, Agarwal P, et al. Liposomes in topical ophthalmic drug delivery: an update. Drug Deliv 2016;**23**(4):1075-91 doi: 10.3109/10717544.2014.943336[published Online First: Epub Date]|.

8. Metselaar JM, Wauben MH, Wagenaar-Hilbers JP, Boerman OC, Storm G. Complete remission of experimental arthritis by joint targeting of glucocorticoids with long-circulating liposomes. Arthritis Rheum 2003;**48**(7):2059-66 doi: 10.1002/art.11140[published Online First: Epub Date]|.

9. Hofkens W, Schelbergen R, Storm G, van den Berg WB, van Lent PL. Liposomal targeting of prednisolone phosphate to synovial lining macrophages during experimental arthritis inhibits M1 activation but does not favor M2 differentiation. PLoS One 2013;**8**(2):e54016 doi: 10.1371/journal.pone.0054016[published Online First: Epub Date]|.

10. Chang DF, Wong V. Two clinical trials of an intraocular steroid delivery system for cataract surgery. Trans Am Ophthalmol Soc 1999;**97**:261-74; discussion 74-9

11. Donnenfeld E, Holland E. Dexamethasone Intracameral Drug-Delivery Suspension for Inflammation Associated with Cataract Surgery: A Randomized, Placebo-Controlled, Phase III Trial. Ophthalmology 2018;**125**(6):799-806 doi: 10.1016/j.ophtha.2017.12.029[published Online First: Epub Date]|.

12. Donnenfeld ED, Solomon KD, Matossian C. Safety of IBI-10090 for inflammation associated with cataract surgery: Phase 3 multicenter study. J Cataract Refract Surg 2018;**44**(10):1236-46 doi: 10.1016/j.jcrs.2018.07.015[published Online First: Epub Date]|.

13. Blizzard C, Desai A, Driscoll A. Pharmacokinetic Studies of Sustained-Release Depot of Dexamethasone in Beagle Dogs. J Ocul Pharmacol Ther 2016;**32**(9):595-600 doi: 10.1089/jop.2016.0025[published Online First: Epub Date]|.

14. Wong CW, Czarny B, Metselaar JM, et al. Evaluation of subconjunctival liposomal steroids for the treatment of experimental uveitis. Sci Rep 2018;**8**(1):6604 doi: 10.1038/s41598-018-24545-2[published Online First: Epub Date]|.

15. van den Hoven JM, Van Tomme SR, Metselaar JM, Nuijen B, Beijnen JH, Storm G. Liposomal drug formulations in the treatment of rheumatoid arthritis. Mol Pharm 2011;**8**(4):1002-15 doi: 10.1021/mp2000742[published Online First: Epub Date]|.

16. Metselaar JM, van den Berg WB, Holthuysen AE, Wauben MH, Storm G, van Lent PL. Liposomal targeting of glucocorticoids to synovial lining cells strongly increases therapeutic benefit in collagen type II arthritis. Ann Rheum Dis 2004;**63**(4):348-53 doi: 10.1136/ard.2003.009944[published Online First: Epub Date]|.

17. Lobatto ME, Calcagno C, Otten MJ, et al. Pharmaceutical development and preclinical evaluation of a GMP-grade anti-inflammatory nanotherapy. Nanomedicine 2015;**11**(5):1133-40 doi: 10.1016/j.nano.2015.02.020[published Online First: Epub Date]|.

18. Jabs DA, Nussenblatt RB, Rosenbaum JT, Standardization of Uveitis Nomenclature Working G. Standardization of uveitis nomenclature for reporting clinical data. Results of the First International Workshop. Am J Ophthalmol 2005;**140**(3):509-16 doi: 10.1016/j.ajo.2005.03.057[published Online First: Epub Date]|.

19. Lorenz K, Dick B, Jehkul A, Auffahrt GU. Inflammatory response after phacoemulsification treated with 0.5% prednisolone acetate or vehicle. Graefes Arch Clin Exp Ophthalmol 2008;**246**(11):1617-22 doi: 10.1007/s00417-008-0908-2[published Online First: Epub Date]|.

20. Agrawal R, Murthy S, Ganesh SK, Phaik CS, Sangwan V, Biswas J. Cataract surgery in uveitis. Int J Inflam 2012;**2012**:548453 doi: 10.1155/2012/548453[published Online First: Epub Date]|.

21. Kim SJ, Equi R, Bressler NM. Analysis of macular edema after cataract surgery in patients with diabetes using optical coherence tomography. Ophthalmology 2007;**114**(5):881-9 doi: 10.1016/j.ophtha.2006.08.053[published Online First: Epub Date]|.

22. Schmier JK, Covert DW, Hulme-Lowe CK, Mullins A, Mahlis EM. Treatment costs of cystoid macular edema among patients following cataract surgery. Clin Ophthalmol 2016;**10**:477-83 doi: 10.2147/OPTH.S98892[published Online First: Epub Date]|.

23. Tan DT, Chee SP, Lim L, Lim AS. Randomized clinical trial of a new dexamethasone delivery system (Surodex) for treatment of post-cataract surgery inflammation. Ophthalmology 1999;**106**(2):223-31 doi: 10.1016/S0161-6420(99)90060-X[published Online First: Epub Date]|.

24. Boyer DS, Yoon YH, Belfort R, Jr., et al. Three-year, randomized, sham-controlled trial of dexamethasone intravitreal implant in patients with diabetic macular edema. Ophthalmology 2014;**121**(10):1904-14 doi: 10.1016/j.ophtha.2014.04.024[published Online First: Epub Date]|.

25. Tyson SL, Bafna S, Gira JP, et al. Multicenter randomized phase 3 study of a sustained-release intracanalicular dexamethasone insert for treatment of ocular inflammation and pain after cataract surgery. J Cataract Refract Surg 2019;**45**(2):204-12 doi: 10.1016/j.jcrs.2018.09.023[published Online First: Epub Date]|.

26. Lee SY, Chee SP, Balakrishnan V, Farzavandi S, Tan DT. Surodex in paediatric cataract surgery. Br J Ophthalmol 2003;**87**(11):1424-6 doi: 10.1136/bjo.87.11.1424[published Online First: Epub Date]|.

27. Tan DT, Chee SP, Lim L, Theng J, Van Ede M. Randomized clinical trial of Surodex steroid drug delivery system for cataract surgery: anterior versus posterior placement of two Surodex in the eye. Ophthalmology 2001;**108**(12):2172-81

28. Tugal-Tutkun I, Herbort CP. Laser flare photometry: a noninvasive, objective, and quantitative method to measure intraocular inflammation. Int Ophthalmol 2010;**30**(5):453-64 doi: 10.1007/s10792-009-9310-2[published Online First: Epub Date]|.

29. De Maria M, Iannetta D, Cimino L, Coassin M, Fontana L. Measuring Anterior Chamber Inflammation After Cataract Surgery: A Review of the Literature Focusing on the Correlation with Cystoid Macular Edema. Clin Ophthalmol 2020;**14**:41-52 doi: 10.2147/OPTH.S237405[published Online First: Epub Date]|.

30. Liu Y, Luo L, He M, Liu X. Disorders of the blood-aqueous barrier after phacoemulsification in diabetic patients. Eye (Lond) 2004;**18**(9):900-4 doi: 10.1038/sj.eye.6701349[published Online First: Epub Date]|.

9

Summary and discussion

Summary and discussion

Ocular drug delivery remains a unique challenge due to the complex anatomical barriers in the eye, which, while maintaining an immune-privileged intraocular environment that is vital for its normal function, also limits drug bioavailability during disease states. The tear film, conjunctiva, cornea, sclera, the blood-aqueous barrier, and the blood-retinal barrier are the main penetration barriers to the anterior and posterior segments of the eye.[1] Since the dawn of ophthalmic therapeutics, eyedrops have been the standard for the treatment of anterior segment diseases. As discussed in Chapter 2, the bioavailability of topically administered eyedrops is poor (less than 5%). Reliance on patient compliance further compounds the problem of low bioavailability. Further, instilling eye drops is a challenging task for most people, especially for children, the elderly, and patients with physical or mental disabilities. For decades, patients and ophthalmologists have come to accept these shortcomings as an unavoidable part of the treatment of anterior segment eye conditions.

Intravitreal administration of drugs such as anti-VEGF agents and corticosteroids is the mainstay of treatment for many posterior segment diseases. There are, however, drawbacks to the way these drugs are delivered. Their relatively short ocular half-lives relative to the duration of the illness necessitates frequent repeated injections into the eye. Regular injections result in a cumulatively high risk of inadvertent trauma to intraocular structures and severe blinding infections, a heavy treatment burden, and escalating costs. Compliance is also an issue, as frequent injections and clinic visits lead to patient fatigue that erode treatment benefits.[1]

Nanomedicines represent a class of advanced drug delivery systems that can potentially increase the therapeutic index of many drug molecules.[2] They have the potential to usher in a new era for ophthalmic therapeutics by replacing eyedrops as the primary mode of treatment for anterior segment diseases and reducing reliance on intravitreal administration for posterior segment diseases. If this potential can be realized, it would entail a paradigm shift in the therapeutic approach to eye diseases.

In this Ph.D. thesis, we explored the use of liposomes as a drug delivery system for corticosteroids to treat ocular inflammation, the most common key factor underlying ocular pathology using anterior uveitis and proliferative vitreoretinopathy (PVR) animal models, representing anterior segment and posterior segment inflammation, respectively, were chosen to test our hypothesis that liposomal steroids are effective and can provide sustained anti-inflammatory action for ocular inflammatory diseases Our promising preclinical results culminated in a first in man, Phase I/II clinical trial that demonstrated the safety and efficacy of a single dose of liposomal steroid, administered subconjunctivally, replacing eyedrops as anti-inflammatory treatment after cataract surgery.

The large interest garnered by nanomedicine as drug delivery systems in oncology has spilled over to the ophthalmic space. In Chapter 2, we presented an overview of the landscape for drug delivery systems (DDS) in the treatment of anterior segment inflammation. The various DDS for anti-inflammatory drugs for the treatment of anterior segment inflammation are listed and summarized in this review, with a focus on commercially available products and those in clinical trials or late-stage preclinical development. Dextenza, INVELTYS, Dexycu, and Bromsite are examples of DDS that have enjoyed success in clinical trials leading to FDA approval.

In Chapter 3, we present an overview of the DDS for posterior segment drug delivery. Inhibitors of vascular endothelial growth factors and corticosteroids are the most commonly used therapeutics for posterior segment eye diseases. Although effective, the need for frequent and invasive intravitreal injections limits the long term utility of these interventions. The quest for sustained action therapeutics that can be delivered to target tissue in the least intrusive manner is an arduous endeavour that has ended in premature failure for several technologies in Phase 2 or 3 trials. Nevertheless, there have been promising preclinical studies, and more are on the horizon. Port delivery systems for the treatment of exudative age related macular degeneration (AMD) have entered Phase 3 trials, and a wide array of preclinical studies have demonstrated the potential for nanoparticles to deliver therapeutics into the posterior segment via minimally invasive routes.[1]

Liposomes are lipid vesicles that can encapsulate drug molecules, for protecting the drug from degradation and improving penetration of the drug into the eye. Liposomes as a drug delivery system may help to overcome the problems associated with eye drops. In Chapter 4, we studied the effectiveness of a formulation of liposomal steroids, administered as a single injection in the subconjunctival space, for the treatment of experimental uveitis in rabbit eyes. After induction of uveitis (Day 0), rabbits were allocated to one of 5 treatment groups and received treatment corresponding to the allocation on Day 3. The treatment groups were: a single dose of subconjunctival liposomal steroids (group 1: prednisolone phosphate and group 2: triamcinolone acetonide), group 3: steroid eyedrops four times a day, group 4: a single dose of subconjunctival free steroid and group 5: no treatment. Rabbits that received a single injection of subconjunctival steroids had significantly lower inflammatory scores (3-6 fold greater reduction in inflammatory score) than untreated rabbits on Day 4 and Day 8 after induction of uveitis, and 2-fold greater reduction in inflammatory score than rabbits given steroid eye drops four times a day for 14 days on Day 8 ($p=0.03$). The subconjunctival liposomal steroid groups continued to have more significant suppression of inflammation than untreated rabbits after a repeat induction of uveitis on Day 8. Immunohistochemical analyses showed that liposomes accumulated in the iris and ciliary body (the primary inflamed sites in anterior uveitis), were also found within macrophages and persisted in the eye for at least one month. This study demonstrates that a single subconjunctival injection of liposomal steroids was effective for the treatment of experimental uveitis and provided sustained anti-inflammatory action that can potentially replace eyedrops as the standard treatment for anterior segment inflammation.[3]

Following the preclinical success of liposomal steroid in treating anterior segment ocular inflammation, we endeavored to achieve the same for posterior segment inflammatory diseases. In Chapter 5, we evaluated the suitability of a previously described rabbit model of PVR as a preclinical model of posterior segment inflammation to test the effectiveness of liposomal steroids. Because PVR is a complex disease with multiple disease-causing pathways, including inflammation mediated by

macrophages and glial cells and epithelial-mesenchymal transition, we sought to measure the levels of pro-inflammatory cytokines and growth factors involved at various time points in the evolution of PVR, to determine an optimal time for administering anti-inflammatory treatment. With this model, we found that PVR began as early as two weeks after surgery, with about 50% of eyes developing severe PVR with retinal detachment at week 4. Our findings demonstrate that inflammation (as represented by elevation of IL-8) spikes within the first two weeks and continues to persist up to 4 weeks after induction of the PVR process. CRP and IFN-γ were significantly associated with PVR severity, suggesting that inflammation not only incites the PVR process but also perpetuates its severity. From these results, we postulate that anti-inflammatory treatment has to be initiated early in the PVR process and should be sustained for at least four weeks to suppress the development of severe PVR.[4]

Having studied the pathogenesis and timing of anti-inflammatory treatment in Chapter 5, we were unsatisfied with the low reproducibility of the rabbit PVR model. An ideal animal model of PVR should have an anatomy and physiology that is as close to the human eye as possible. Compared to the rabbit eye, the pig eye has an anatomy that is closer to that of the human eye and therefore has the potential to be more representative of the human disease. Thus, we decided to switch to the pig model with an additional aim of refining the reproducibility of the PVR model. The pig as a representative model for PVR was first described by García-Layana et al. in 1997 as an improvement to the rabbit model.[5] In Chapter 6, we compared two surgical models of PVR in the minipig eye: a previously described model using exogenous retinal pigment epithelial (RPE) cells derived from cadaveric pig eyes, and a new modified model that we designed using native (autologous) RPE cells[6] to induce PVR disease process in a manner that was as similar as possible to the disease process in human eyes. We found that this new model was 1.67 times more likely to simulate severe PVR than the previously described model (50% vs. 83.3%). This new model is a consistent model of PVR in the minipig that can be surgically induced using native RPE cells and may be a suitable model for both understanding the pathogenesis of PVR and for testing of novel therapeutics to treat PVR.

Having developed a representative PVR model and identified the optimal timing and duration of anti-inflammatory treatment, we were ready to determine whether liposomal steroids could be an effective treatment for PVR. Instead of subconjunctival injection, we took the intravitreal administration route, as we did not expect that subconjunctival injection could yield satisfying results in case of posterior segment disease. To effectively manage PVR, therapeutic drug levels will have to be sustained in the retina. After subconjunctival injection, the sclera acts as an ocular barrier against drug penetration, resulting in lower drug levels in the inflamed posterior segment compared to intravitreal injection. Previous studies have shown that the intravitreal half-life is longer for drugs injected intravitreally compared to subconjunctival injection.[7, 8] We considered that liposomal encapsulation of glucocorticoids can provide several benefits, including prolonged residence time, selective anti-inflammatory action on macrophages, one of the main protagonists in PVR, and avoidance of toxic effects from unintended action at non-inflamed sites. In Chapter 7, we describe the results of a preclinical study to test the efficacy of an intravitreal injection of liposomal steroid, given immediately after PVR induction, in mitigating the disease severity of PVR. This is the first study evaluating the effectiveness of liposomes as a drug delivery system for steroids to treat PVR. Eyes that received liposomal prednisolone phosphate were five times less prone to develop retinal re-detachment from severe PVR and had significantly lower median PVR severity at four weeks after PVR induction. Further studies are needed to validate these findings and to evaluate optimal dosing as well as different routes of administration. At the same time, liposomal drug delivery systems can potentially reduce the retinal toxicity of anti-proliferative therapeutics, bringing about the possibility of combining the synergistic effects of steroid with anti-proliferative therapeutics such as daunomycin for the treatment of PVR.

In Chapter 8, we present the results of a Phase I/II clinical trial that evaluated the safety and efficacy of a single subconjunctival dose of liposomal prednisolone phosphate in suppressing post-cataract surgery inflammation. The proportion of patients with resolution of clinically visible inflammation was 0%, 80%, 80%, and 100% at day 1, week 1, month 1 and month 2 after cataract surgery, respectively. No ocular or non-ocular adverse events were observed. These results are encouraging

and suggest that the subconjunctivally delivery of steroids with a liposomal drug delivery system could potentially replace eyedrops as anti-inflammatory therapy following cataract surgery.

Conclusions and perspectives

The use of eyedrops for ocular disease treatment dates back to the 1870s, when pilocarpine eyedrops were used to treat glaucoma.[9] Despite their shortcomings, topical steroid eye drops are still the most widely used treatment for anterior chamber inflammatory diseases. This is set to change in the near future, driven by advances in healthcare delivery, nanomedicine, and patient expectations. Corticosteroids and other anti-inflammatory drugs, delivered by the ideal DDS, should provide targeted and sustained drug delivery to inflamed tissue while avoiding off-target effects, particularly in the trabecular meshwork, which often leads to raised intraocular pressure and glaucoma. The drug delivery system itself should be well tolerated both in terms of the way delivery is performed (non-invasive) and in terms of ocular adverse events. There is high clinical demand, not just for post ocular surgery inflammation, but also for post corneal transplant patients that require long term steroid use. These patients are often suffering from strong inflammation ongoing particularly in the anterior segment of the eyes after completion of the surgical procedure, and will greatly benefit from an improvement in the therapeutic index of ocular steroids. DDS have the potential for sustained delivery of the lowest dose of steroid non-invasively and without reliance on patient compliance. However, commercially available ocular DDS therapies such as Dextenza and Dexycu are still not widely accepted by patients and the ophthalmic community. Possible reasons for the low adoption rate include the need for invasive intraocular injection into the anterior chamber (Dexycu), and intracanalicular implants may cause trauma to the tear duct and could be dislodged prematurely (Dextenza). Liposomal drug delivery systems may have several advantages, including a less invasive route of administration, sustained action with a single subconjunctival injection thereby eliminating patient compliance issues, and targeted delivery of steroid directly to inflamed tissue with minimal release of free drug elsewhere thereby reducing unwanted off-target side effects. Our Phase I/II

clinical trial demonstrated the safety and efficacy of liposomal prednisolone phosphate for the treatment of post-cataract surgery inflammation. Some questions, such as the dosing requirements for patients at risk for more prolonged inflammation, remain. These will be addressed in a larger phase II/III clinical trial. Liposomal DDS injected by the physician subconjunctivally at the time of ocular surgery may be an ideal method to deliver anti-inflammatory therapy to the front of the eye, ushering in a new era of "dropless" ocular therapeutics for ocular inflammation.

For posterior segment therapeutics, the next major step is to develop sustained action therapeutics that can be delivered to target tissue in the least invasive manner. This is an arduous task, and many promising technologies have met with premature failure in Phase 2 or 3 trials. Nevertheless, there have been preclinical successes taken into clinical development, and more are on the horizon: implantable sustained-release corticosteroid formulations already exist for the treatment of diabetic macular edema (DME), while port delivery systems for the treatment of exudative AMD have entered Phase 3 trials. A wide array of preclinical studies have demonstrated the potential for nanoparticles and cell penetrating peptides to deliver therapeutics into the posterior segment via minimally invasive routes but translation into human trials remain ponderous and uncertain at every stage toward clinical development. It is a testimony that better, more clinically effective therapies and sustained delivery systems should remain a focused area in the advancement of clinical treatment of posterior segment diseases. PVR is the quintessential example of a posterior segment disease that may benefit from a nanomedical DDS. While inflammation is a dominant process in PVR, it is surprising that a sizable number of studies and clinical trials on novel anti-inflammatory and anti-proliferative drugs have found generally only small effect sizes for improving therapeutic outcomes in patients with PVR. Contrary to these studies, we found a significant treatment benefit in initiating early anti-inflammatory treatment delivered by a liposomal DDS in a preclinical model.

We postulate that our liposomal steroid formulation may exert its therapeutic effect in the following ways. First, prednisolone phosphate given early in the inflammatory phase of PVR keeps inflammatory pathways in check thus preventing 'runaway inflammation' from igniting the PVR

cascade. Second, encapsulation of steroid within liposomes enhances their dwell time in the vitreous cavity, extending their therapeutic effect well beyond the inflammatory phase of PVR. Finally, liposome encapsulation of prednisolone phosphate alters their site of action from T cells to predominantly macrophages, the major inflammatory cell type in PVR, and shifts their phenotype from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype, thereby tilting the balance back in favor of retinal repair rather than the over-exuberant scarring seen in PVR. Further studies are needed to fully explore and elaborate these mechanisms.

In conclusion, we demonstrated the effectiveness of a single dose of liposomal DDS in delivering steroid for both anterior and posterior segment indications. Liposomal steroids can be used as an anti-inflammatory treatment after any form of ocular surgery, eliminating the problems of eyedrops with the use of a single subconjunctival injection. We anticipate that this liposomal DDS can also be used for other ocular indications where persistent inflammation requires long term and intensive use of steroid eyedrops such as anterior uveitis, post glaucoma surgery and corneal graft surgeries. For retinal diseases, we expect advantages over current conventional treatment with intravitreal antivascular endothelial factors (longer sustained action) and intravitreal steroid implants e.g. Ozurdex (less side effects). Further studies are needed to validate these findings, optimize the dose, evaluate different routes of administration and to evaluate potential synergistic effects of liposomal steroid with anti-angiogenic and anti-proliferative therapeutics for the treatment of posterior segment diseases.

References

1. Wong CW, Wong TT. Posterior segment drug delivery for the treatment of exudative age-related macular degeneration and diabetic macular oedema. *Br J Ophthalmol* 2019;103:1356-1360.

2. Shi J, Kantoff PW, Wooster R, Farokhzad OC. Cancer nanomedicine: progress, challenges and opportunities. *Nat Rev Cancer* 2017;17:20-37.

3. Wong CW, Czarny B, Metselaar JM, Ho C, Ng SR, Barathi AV, Storm G, Wong TT. Evaluation of subconjunctival liposomal steroids for the treatment of experimental uveitis. *Sci Rep* 2018;8:6604.

4. Wong CW, Cheung N, Ho C, Barathi V, Storm G, Wong TT. Characterisation of the inflammatory cytokine and growth factor profile in a rabbit model of proliferative vitreoretinopathy. *Sci Rep* 2019;9:15419.

5. Garcia-Layana A, Pastor JC, Saornil MA, Gonzalez G. Porcine model of proliferative vitreoretinopathy with platelets. *Curr Eye Res* 1997;16:556-563.

6. Umazume K, Barak Y, McDonald K, Liu L, Kaplan HJ, Tamiya S. Proliferative vitreoretinopathy in the Swine-a new model. *Invest Ophthalmol Vis Sci* 2012;53:4910-4916.

7. Gough G, Szapacs M, Shah T, Clements P, Struble C, Wilson R. Ocular tissue distribution and pharmacokinetic study of a small 13kDa domain antibody after intravitreal, subconjuctival and eye drop administration in rabbits. *Exp Eye Res* 2018;167:14-17.

8. Nomoto H, Shiraga F, Kuno N, et al. Pharmacokinetics of bevacizumab after topical, subconjunctival, and intravitreal administration in rabbits. *Invest Ophthalmol Vis Sci* 2009;50:4807-4813.

9. Komatsu Y. [A history of the development of eye drops used to treat glaucoma]. *Yakushigaku Zasshi* 2007;42:7-16.