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INVESTIGATION OF KINETICS OF METHOTREXATE FOR THERAPEUTIC TREATMENT OF INTRAOCULAR LYMPHOMA

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By

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ABSTRACT

Primary intraocular lymphoma (PIOL) is a term that refers to non-metastatic malignant lymphoid neoplasia that arises primarily within the eye. One of the currently available treatment options for PIOL is intravitreal injection of methotrexate. The main objective of this study is to numerically investigate the kinetics of methotrexate in a human eye following the standard induction–consolidation–maintenance (I-C-M) injection protocols for the treatment of PIOL (numerical study). In addition, we investigated the intravitreal concentration of methotrexate over time, and toxicity associated with slow sustained release of the drug from a biodegradable device containing methotrexate implanted in a deep scleral pocket of the eyes of normal rabbits (*in vivo* study).

The 3-dimensional model of a rabbit eye was adapted from our previous studies and a human eye model was constructed based on its physiological dimensions. The retinal permeability values of methotrexate calculated for an albino rabbit and human eye were 1.1×10^{-5} cm/s and 9.25×10^{-6} cm/s, respectively. Given that the dosage above $0.1 \mu\text{M}$ is considered tumoricidal, the cytotoxic levels of methotrexate were consistently achieved only in the induction phase and the concentration levels dropped below the cytotoxic levels for part of the consolidation and maintenance phases of the treatment following the I-C-M protocols. By making use of the results of this study, one could select a dosing interval for serial injections of methotrexate or establish a treatment schedule using controlled release methotrexate implant for the treatment of PIOL.

For the *in vivo* study, biodegradable micro-needle implants (approximately 8 mg) loaded with 10% wt of methotrexate were fabricated using solvent cast method. The implants were all appropriately rigid at room temperature or when cooled in a refrigerator following fabrication. The micro-needles were expected to penetrate the inner scleral lamellae in the scleral pocket and the underlying uveal tissue of the ciliary body and project into the peripheral vitreous. All the implants were inserted surgically in a deep lamellar scleral pocket created in each eye of three albino New Zealand rabbits. Unfortunately, during the surgery unanticipated softening of the micro-needle implant occurred when the implant came in contact with the ocular tissues at body temperature. Consequently, the implant was not placed in the intended position but was simply placed in the intrascleral pocket. The micro-needle implants loaded with methotrexate did not perform mechanically in the intended way.

The animals were sacrificed four weeks after the surgical implantation and the eyes were enucleated. The histopathology results showed that the softened implants (without and with the medication) placed in the intrascleral space (with none of its needles penetrating any eye wall structures) did not cause any obvious major toxicity to the eye. Since the micro-needles of the bio-degradable implant did not perform as expected because of the heat induced softening, our group is currently considering alternative implant designs and fabrication procedures which not only offer improved mechanical strength (rigidity) at higher temperatures but also allow us to place the implant directly in the vitreous via a full thickness eye wall microincision.

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TABLE OF CONTENTS

List of Tables.....	vii
---------------------	-----

List of Figures.....	viii
----------------------	------

Chapter 1: Introduction

1.1: Motivation and significance of this study.....	1
1.2: Outline of Thesis.....	7

Chapter 2: Background

2.1: Structure of Eye.....	9
2.2: Posterior segment components.....	10
2.3: Anterior segment components.....	13
2.4: Aqueous humor hydrodynamics.....	14
2.5: Drug delivery techniques.....	16
2.6: Diffusion and convection.....	19

Chapter 3: Numerical Investigation of Kinetics of Methotrexate in a Human Eye

3.1: Methodology: Numerical Study.....	21
3.1.1: Model development and conservation equations.....	21
3.1.2: Model parameters	29
3.1.2: Solution methodology.....	30
3.2: Results: Numerical Study.....	35
3.2.1: Retinal permeability and half life of methotrexate in rabbit and human eyes.....	35
3.2.2: Elimination of methotrexate in rabbit and human eyes following an intravitreal injection.....	37

3.2.3: Methotrexate concentration in human eye following standard Induction–Consolidation–Maintenance (I-C-M) protocols.....	44
3.2.4: Kinetics of methotrexate in human eye following different doses of intravitreal injection.....	47
3.2.5: Therapeutic release rate of a 90-day sustained-release methotrexate implant.....	50
3.3: Discussion.....	52
Chapter 4: Toxicity Assessment of a Biodegradable Implant Loaded with Methotrexate in Normal Rabbit Eye	
4.1: Material and Methods: <i>in vivo</i> study.....	58
4.1.1: Design and Device Fabrication.....	58
4.1.2: Device Implantation.....	62
4.1.3: Histopathology Study.....	63
4.2: Results: Histopathological findings.....	65
4.3: Discussion.....	69
Chapter 5: Conclusion and Future Study.....	72
References.....	75

LIST OF TABLES

Table I. <i>In vivo</i> studies used for the estimation of retinal permeability of MTX in rabbit and human eyes.....	26
Table II. Duration time for which therapeutic MTX levels were achieved in the vitreous of human eye following different injection doses.....	49

LIST OF FIGURES

Figure 1: (A) Fundus photo showing primary intraocular lymphoma (PIOL)	
(B) Photomicrograph slide showing lymphoma between retinal and choroid.....	2
Figure 2: (A) Intravitreal Injection and (B) Intravitreal Implant.....	6
Figure 3: Anatomy of Eye.....	9
Figure 4: (A) Sclera and choroid when compared to the retina (layers)	
(B) Anatomy of retina and posterior eye.....	12
Figure 5: Aqueous Humor.....	15
Figure 6: Drug delivery techniques used for the treatment of intraocular diseases.....	17
Figure 7: Typical drug concentration profile in the vitreous following an	
Intravitreal injection.....	18
Figure 8: Cross sectional view of the numerical models of (A) Human eye and	
(B) Rabbit eye with the drug site positioned closer to the hyaloid membrane.....	22
Figure 9: Compartments of the eye models:	
(A) Sclera (B) Choroid(C) Retina-Choroid (D) Vitreous.....	23
(E) Lens (F) Schlemm’s Canal (G) Hyaloid Membrane (H) Posterior Chamber..	24
(I) Iris (J) Injection/Implant Site (K) Anterior Chamber.....	25
Figure 10: Computational grid	
(A) Human Eye.....	33
(B) Rabbit Eye.....	34
Figure 11: Comparison of the model simulated vitreous concentrations with the	
available <i>in vivo</i> and clinical data.	
(A) Numerical eye models were matched with the experimental	

Conditions (injection location, volume and dose).....	36
(B) Comparison of simulated MTX vitreous concentrations and the experimentally determined concentrations.....	36
Figure 12: Elimination characteristics of MTX in human and rabbit eyes following intravitreal injection.....	38
Figure 13: Distribution of MTX in rabbit and human eyes at different time points	
(A) Distribution of MTX at t = 0.02hrs. (A1) Rabbit Eye and (A2) Human Eye.....	39
(B) Distribution of MTX at t = 3hrs. (B1) Rabbit Eye and (B2) Human Eye.....	40
(C) Distribution of MTX at t = 7hrs. (C1) Rabbit Eye and (C2) Human Eye.....	41
(D) Distribution of MTX at t = 24hrs. (D1) Rabbit Eye and (D2) Human Eye.....	42
(E) Distribution of MTX at t = 48hrs. (E1) Rabbit Eye and (E2) Human Eye.....	43
Figure 14: Model simulated kinetics of MTX in a human eye following the Induction–Consolidation–Maintenance (I-C-M) protocols	
(A) Protocol used by De Smet et al.....	45
(B) Protocol used by Frenkel et al.....	46
Figure 15: Mean vitreous concentration of MTX in a human eye following different doses of intravitreal injection.....	48
Figure 16: Mean vitreous concentration of MTX in a human eye following a 90–day controlled release implant with different release rates.....	51
Figure 17: The fabrication procedure used for making the biodegradable polymeric MTX implant based on micromolding technique from high-aspect-ratio poly (dimethylsiloxane) (PDMS).....	60
Figure 18: Biodegradable polymeric MTX implant used for this study	

(A) Design of the implant and	61
(B) Actual MTX implant used in the <i>in vivo</i> experiments.....	61

Figure 19: Implantation procedure employed in the animal experiments

(A) Scleral flap made in the superotemporal quadrant of the eye	
(B) Device implantation and	
(C) Suturing the scleral flap and conjunctival peritomy.....	64

**Figure 20: Histopathologic examination of rabbit eyes four weeks after the
implantation. Photomicrographs of**

(A) Empty intrascleral pocket in the left eye of animal 2 (H&E-20X),	
(B) Foreign body giant cell reaction to suture material in the left eye of animal 3	
(C) Intrascleral pocket containing implant residue in the right eye of animal 2	
(D) Foreign body inflammation observed in the right eye of animal 3.....	68

Figure 21: Intended penetration of the biodegradable micro-needle implant.....73

CHAPTER 1

Introduction

1.1 Motivation and Significance of the Study

Primary intraocular lymphoma (PIOL) is a term that refers to non-metastatic malignant lymphoid neoplasia that arises primarily within the eye. It is to be differentiated from intraocular malignant lymphoid infiltrates and tumors that occur metastatically from a primary systemic non-Hodgkin's lymphoma. An estimated 100 new cases of PIOL were diagnosed in the United States between 2000 and 2005, with a slight male predominance.^{1, 2} PIOL occurs in three major clinical forms: (1) primary vitreo-retinal lymphoma (PVRL), (2) primary uveal lymphoma (PUL), and (3) primary intraocular lymphoma with overlap features (i.e. both vitreoretinal and uveal features).³ PVRL comprises at least 85% of cases of PIOL encountered in the United States. This form of lymphoma represents intraocular involvement by primary central nervous system lymphoma (PCNSL).⁴

The central nervous system lesions associated with PVRL may precede, occur concurrently with, or follow detection of the intraocular lesions. PVRL and PCNSL appear to be independent primary sites and are not considered to be metastatic from one to the other. In studies where adequate tissue has been obtained, immunocytopathologic analysis of PVRL has usually shown the neoplasia to be large cell, B-cell non-Hodgkin's lymphoma.⁵ PVRL presents most commonly as an ocular disorder that simulates intermediate or posterior uveitis with pale, finely-dispersed intravitreal lymphoid cells and/or geographic creamy yellow accumulations of neoplastic lymphoid cells beneath the retinal pigment epithelium as shown in Figs 1A and 1B. Bilateral involvement occurs in up to 80 % of patients with PVRL.⁶ This disorder may affect one or both eyes multicentrically

but can be grossly asymmetric or sequential rather than concurrent and symmetric.

Fig 1: A) Fundus photo showing primary intraocular lymphoma (PIOL);
B) Photo-micrograph slide showing lymphoma between retinal and choroid.

Initial treatment for PVRL depends on whether one or both eyes are involved, whether the brain is also involved at that time, and whether and how prior primary central nervous system lymphoma (if any) had been managed. The two principal treatment options for PVRL/PCNSL are fractionated external beam radiation therapy to both eyes and the whole brain simultaneously and intravenous chemotherapy (with or without concurrent intrathecal chemotherapy). In recent years, some eyes with PVRL (especially ones with recurrent intraocular lymphoma after prior intravenous chemotherapy or ocular irradiation but also some eyes of patients without concurrent demonstrable active PCNSL) have been treated by a course of intravitreal injections of methotrexate.⁷⁻¹⁰

Currently, methotrexate is considered to be one of the most effective and clinically non-toxic agents to treat PIOL. The *in vitro* cytotoxic activity of methotrexate for 63

different cell lines has been evaluated and reported. The therapeutic levels of methotrexate range from 0.1 μ M to 1 μ M, with a mean IC_{50} of 0.32 μ M. Methotrexate interferes with tumor cell growth and specifically interferes with rapidly dividing cells. The exact mechanism is unknown but it acts during the DNA and RNA synthesis phase. Since the half-life of methotrexate is of the order of only a few hours, frequent injections are generally required to achieve prolonged cytotoxic levels.^{8, 11}

Different research groups have used the induction-consolidation-maintenance (I-C-M) injection protocols using methotrexate, with some changes in the dosing intervals for the treatment of PIOL. The time scale at which methotrexate reacts with the tumor cells has not been reported in the literature. However, in the data published from two trials by different groups, it was reported that 95% of the eyes needed 12-13 methotrexate injections or less to be cleared of malignant cells and retinal infiltrates.⁹ The exact number of injections needed for the remission of tumor was patient specific and varied from one patient to the other. Tumor cells in some patients disappeared after 2 injections (one week), and some patients needed 16 injections (2-3 months) for complete remission. From this data, we can conclude that therapeutic levels of methotrexate need to be maintained inside the eye at least for 1 week (i.e. the tumor cells need to be exposed to methotrexate for 1 week), for the eyes to be cleared of malignant cells. These repeated administrations can be associated with ocular complications such as endophthalmitis, cataract, vitreous hemorrhage, and rhegmatogenous retinal detachment.^{5, 9, 12, 13}

Considering the narrow therapeutic range for methotrexate, it is critical to have prior knowledge of drug distribution and its elimination characteristics in order to develop effective treatment protocol for PIOL and maximize the therapeutic benefits. The

distribution of methotrexate in rabbit and human eyes is significantly influenced by its rate of elimination through retina and Schlemm's canal. The value of retinal permeability of methotrexate is not available in the published literature. Thus, in this study, we have numerically calculated the permeability of methotrexate across the retina for rabbit and human eyes by making use of the results of the previous *in vivo* and clinical studies, respectively.^{8, 11} The kinetics of methotrexate was then investigated inside the human eye following the standard induction-consolidation-maintenance (I-C-M) injection protocols. The duration time for which the therapeutic methotrexate levels were maintained in the human eye following different injection doses was calculated and is reported in this thesis. Keeping in mind that repeated injections of methotrexate for two months has induced considerable remission of tumor, a 90-day intravitreal implant was modeled and its mean or average therapeutic release rate was determined in order to have sustained cytotoxic methotrexate levels for three months in vitreous of the human eye.

Sustained-Release-Implantable Devices: A variety of sustained-release drug delivery systems such as liposomes, microspheres, and implantable devices made of biodegradable or non-biodegradable polymers have been investigated by previous authors.¹⁴⁻¹⁷ Over the last two decades such devices have been developed and approved by the US FDA for delivery of prolonged therapeutic levels of ganciclovir in patients with cytomegalovirus retinitis and acquired immune deficiency syndrome, and also other devices have been developed for the treatment of uveitis, proliferative vitreoretinopathy and macular edema.^{16, 18-21} Both intraocular injections and intraocular sustained-release devices are capable of bypassing the blood-retinal barrier (to intraocular drug absorption) as shown in Figs 2A and 2B, respectively. A sustained release device latter also avoids frequent surgical procedures. The drug release kinetics from biodegradable implants has

been studied as a function of blending ratios of the high and low molecular weight polymers, and also various design parameters such as shape, size, and surface area of the implants were investigated.^{19, 22-24}

Most of the drugs exhibit a tri-phasic drug release profile from biodegradable implants. At the beginning, drugs that are deposited at the surface of the implant are released through *initial burst (1st phase)*. This is followed by a slow and *sustained release (2nd phase)* of drug attributed to the diffusion of drug through the polymer. Finally the rapid release of drug or the *late burst (3rd phase)* is caused by the swelling and disintegration of the polymeric implant.²⁴ Ideally, a sustained release device should have a lower initial burst and deliver drug such that the therapeutic levels are achieved for 3-6

Fig 2: A) Intravitreal Injection and B) Intravitreal Implant; (Source: <http://jirehdesign.com/EyeIllustrations/pop/img/MacugenAnim.jpg>)

months. Recent studies have shown that the first burst can be reduced and the second burst can be prevented for implants prepared with a blend of high and low molecular weight polymers.²⁴ No studies have been reported to date of an intravitreal sustained-release device containing methotrexate for treatment of PIOL.

PIOL provides a diagnostic and therapeutic challenge because of its non-specific ocular symptoms, diverse clinical picture, nonspecific presentation, and variable clinical course.^{5, 12, 25} For optimal treatment of PIOL, therapeutic levels of methotrexate need to be maintained in the eye for a prolonged period while avoiding toxic and sub-therapeutic levels. A well-tolerated controlled drug release device has the potential to maintain a sufficient concentration of drug in the eye for a period of time sufficient to eradicate the target disease while avoiding undesirable and toxic peak concentrations of the drug.^{24, 26, 27} Our group designed a new type of biodegradable intraocular implant for intended treatment of selected patients with PIOL. The design and fabrication of the biodegradable micro-needle intraocular implant is reported in this thesis. Furthermore, the safety of a biodegradable ocular micro-needle implant loaded with methotrexate was investigated in a normal rabbit eye.

1.2 Organization of Thesis

Chapter 1 introduces the numerical and the experimental (*in vivo*) research presented in this thesis, while discussing the main motivation of this study. Chapter 2 provides the background on anatomy of the eye and the mode of drug transport inside the eye. In Chapter 3, the geometry of the computational eye models, conservation equations and the boundary conditions used for the numerical calculations are explained. The kinetics of MTX in a human eye following the I-C-M injection protocols and sustained

release implant are explained. Limitations of the numerical study are also discussed in this chapter. Chapter 4 provides the design and fabrication of our biodegradable implants loaded with methotrexate, the implantation procedure employed in our study animals, and the clinical and histopathology studies performed to evaluate the safety of these devices. In addition, the main outcomes of the *in vivo* study and the significance of this work in developing effective treatment strategies for PIOL are discussed in detail.

The conclusions and future recommendations are summarized in Chapter 5. The references are provided at the end of the document.

CHAPTER 2

Background Information

2.1 Structure of the Eye

The eye is generally divided into two segments – anterior and posterior segments as shown in the Fig 3. The cross sectional view of the eye shows three different layers: The external layer formed by the sclera and cornea, intermediate layer, divided into two parts: anterior (iris and ciliary body) and posterior (choroid), and the internal layer, the retina. The eye is filled with two liquids: aqueous humor and vitreous humor. These fluids provide nourishment to the other cells in the eye similar to the way blood vessels provide nourishment to most cells in the body.

Fig 3: Anatomy of Normal Eye; (Source: <http://www.informaworld.com/ampp/image>)

2.2 Posterior Segment Components

The posterior segment is the back two-thirds of the eye. It lies behind the aqueous-filled anterior segment and is separated from it by the lens and zonule. It mainly consists of vitreous, retina, choroid and sclera as shown in Fig 3. The ciliary body is considered to be part of the anterior segment. The delivery of therapeutic doses of drugs to the tissues in the posterior segment of the eye remains a significant challenge. Most of the ocular diseases including diabetic retinopathy, glaucoma, and retinitis pigmentosa, which account for most cases of irreversible blindness cases, affect the posterior segment of the eye.

Vitreous is a colorless, transparent gel consisting of 98% water that fills the eyeball between the lens and the retina. The spherical shape of the eyeball is maintained partly by the vitreous. As one gets old, the vitreous body shrinks in volume. This decrease in size may detach it from the retina and is referred to as vitreous body detachment which in by itself is not a very dangerous phenomenon. But at times when the vitreous body drags along the retina, complications like retinal detachment might occur. The vitreous body is encased in a clear hyaloid membrane similar to the outer membrane seen in the egg white.

Sclera is the tough, fibrous outer layer of the eyeball that forms the whites of the eyes, and is continuous with the cornea as shown in Fig 4A. It is mainly composed of composed of collagen, and a sparse population of fibroblasts, proteoglycans, and a few elastic fibers. The sclera, being rugged and robust, protects the inner, more sensitive parts of the eye like the retina and choroid. The episclera, which is located on top of the sclera, contains blood vessels that nourish the sclera with oxygen and nutrients. Transscleral drug delivery to posterior segment targets have been investigated because the sclera is significantly more permeable than the cornea.²⁸

Choroid is a thin vascular pigmented tissue that lines the inner surface of the sclera as shown in Fig 4A. It supplies oxygen and necessary nutrients to the retina. The macula and the anterior part of the optic nerve are dependent on blood supply from the choroid. The retinal pigment epithelium (RPE) is located between the choroid and the retina whereas the space between the choroid and the sclera is referred to as suprachoroidal space. The choroid has a vast network of blood capillaries and most of the drug that reaches choroid will be cleared through the blood capillaries.

Retina occupies the internal space of the posterior portion of the eye. It is a multi-layered light sensitive tissue as shown in Fig 4B. The outer layer (adjacent to the choroid) of the retina comprises of the retinal pigmented epithelium (RPE) and the inner layer of retina contains two kinds of light receptors, the cones and the rods.^{28, 29} The center of the posterior part of the retina is called Macula. It is responsible for absorbing the strong light (high visual resolution). Most of the systemic drugs administered will not reach the retina, because of the blood-retinal-barrier (BRB) which reduces the penetration of the drug. The BRB poses a biggest limitation to the systemic administration of drugs used for targeting the retina. The tight junctions of the RPE restrict the paracellular transport of polar solutes across the RPE from the choroid to the surface of the retina.

There is a body of experimental evidence supporting the idea that there is active as well as passive transport of drugs out of the vitreous. Previous studies have investigated the inward and outward retinal permeability and attributed an active efflux mechanism for higher outward permeability. The retinal permeability is defined as

$$\text{Retinal Permeability (RP)} = \text{Diffusivity of retina (D}_R\text{)}/\text{thickness of retina (t)}$$

A

B

Fig 4: (A) Sclera and choroid when compared to the retina. (Source: <http://www.cytochemistry.net>);

(B) Anatomy of retina and posterior eye. (Source: <http://www.lef.org/magazine/mag2004/images>)

2.3 Anterior Segment Components

The anterior segment mainly comprises of conjunctiva, cornea, uvea (iris, ciliary body), lens, posterior and anterior chambers. It essentially is the front third of the eye and includes all the structures anterior to vitreous humor.

Conjunctiva is a thin transparent mucous membrane that covers the white part of the eye and lines the eyelids. The eyeball layer is referred to as bulbar conjunctiva and the lining of the eyelids is called palpebral conjunctiva. The transitional region, where the palpebral conjunctiva is reflected to become the bulbar conjunctiva forms a conjunctival sac, (unattached to the eyelids or the eyeball) called as fornix. The conjunctiva is loosely attached to the underlying sclera.

Cornea is the transparent front part of the eye that covers the iris, pupil, and anterior chamber. It is the main structure responsible for light refraction, and its curvature is greater than the rest of the globe. At the circumference of the cornea is the limbus, where the cornea becomes continuous with the sclera and the conjunctiva. Cornea is exposed to the outside environment and therefore must repair itself rapidly. It is composed of five layers of tissues: epithelium, Bowman's membrane, stroma, Decement's membrane, and endothelium. To administer therapeutic agents into the eye through the cornea (using topical eye drops), the drug carriers should be sufficiently small and possess both hydrophilic and lipophilic properties to pass through the tight junctions.

Iris is a thin, circular, contractile diaphragm that controls the light refraction through the lens, depending on the environment. The iris (pigmented) is what determines the eye color. The more melanin pigment found in the iris, the darker the eye color will be.

The iris contains circular and radial muscles. This circular muscle controls the size of the pupil depending on the brightness in the environment. With strong light, the iris sphincter muscles will contract the pupil. In darkness, the dilator muscles of the iris will open the pupil. The pupil diameter decreases when focusing on the objects closer to the eye and increases when focusing on distant objects. This is a reflex known as the Accommodation Reflex.

Ciliary body is extremely important to the health and function of the eye, as it is the source of the nutrient fluid called aqueous humor. The ciliary body contains the ciliary muscle, the muscle that controls the vision accommodation reflex. For nearer objects the lens is increasingly rounded by ciliary muscle contraction, which relaxes the suspensory ligament. When the ciliary muscles are relaxed, the perimeter of the lens is pulled outwards giving it a flattened shape.

Lens is a clear, flexible structure that changes shape and directs the light beam to focus on objects at varying distances. It is suspended by zonule fibers which are attached to the anterior portion of the ciliary body. Se ligaments or zonule fibers contract or relax, thus changing the shape of lens, to accommodate near or distant viewing. This process is called accommodation and it helps in forming a sharp image in retina.

2.4 Hydrodynamics of Aqueous Humor

Aqueous humor contained in the anterior chamber and posterior chamber of the eyes. It is a cleary-water fluid secreted by the ciliary processes of the ciliary body which supplies nutrients to lens and cornea. As shown in Fig 5, the aqueous humor flows through

the space between the iris and the lens (posterior chamber). It flows into the anterior chamber and towards schlemms's canal. Most of the aqueous humor is drained out through the schlemm's canal (trabecular outflow) while the remaining is drained out through sclera (uveosclera outflow). Some part of the fluid generated by the ciliary process flows through the vitreous and across the retina (vitreous outflow). Any abnormalities in the drainage system at the outflow channel results in the increase of the intraocular pressure.

Fig 5: Hydrodynamics of Aqueous Humor

(Source: http://www.medrounds.org/glaucoma-guide/uploaded_images/fig3-2-799969.jpg)

2.5 Drug Delivery Techniques

In this section, various drug delivery techniques used for the treatment of the eye diseases are briefly explained.

Following a systemic administration, drug goes throughout the body, usually carried in blood stream. Generally, the drug will be either injected into the vein or into a muscle. The systemic medications are subjected to the blood-retinal barrier, as it reaches the chorioretinal tissue via the blood circulation. Thus, frequent doses of systemically administered medications are needed to achieve cytotoxic levels inside the eye. However, high systemic doses are associated with serious side effects.

Topical administration is the most commonly used method to administer medication to the eye. It is shown in Fig 6A. The usefulness of topical eye drops is limited by solution drainage, tear dilution, tear turnover, conjunctival vasculature absorption and the corneal epithelium barrier. Generally, less than 5% of a topically applied drug permeates the cornea and reaches intraocular tissues.

Controlled release devices helps in maintaining therapeutic drug levels inside the eye, while avoiding toxic or subtherapeutic levels. The choice of the device design and the implantation site in the eye depends on the location of the disease. Periocular drug delivery techniques such as subconjunctival implant, suprachoroidal implant, and sclera plug offer the advantage of a noninvasive approach. A sustained release device inserted through sclera as shown in Fig 6B is called a sclera plug. A subconjunctival implant is a device placed under a conjunctiva of an eye as shown in Fig 6C. The Fig 6D shows a suprachoroidal implant that is placed inside the suprachoroidal space i.e a potential space

located between the choroid and the sclera. Sustained release device placed inside the vitreous as shown in the Fig 6E is referred to as an intravitreal implant.

Fig 6: Drug delivery techniques used for the treatment of intraocular diseases. (A) Topical Eye drops; (B) Scleral plug; (C) Subconjunctival implant placed under a conjunctiva; (D) Suprachoroidal implant that is placed inside the space located between the choroid and sclera; (E) Intravitreal implant; (F) Intravitreal injection;

(Source: <http://www.informaworld.com/ampp/image?path=/713172959/713554097/F0002.png>)

Intravitreal injection, as shown in Fig 6F, provides the most direct approach to delivering therapeutic drug levels to the tissues of the posterior segment. Typical drug concentration inside the vitreous following an intravitreal injection is shown in Fig 7. As shown in the Fig 7, higher peak concentrations are toxic to the intraocular tissues. But given the short half life of most drugs injected into the vitreous, frequent injections 1–3 times per week are necessary to maintain therapeutic drug levels. Intravitreal injections are very useful in treating acute bacterial infections of the eye that may require one or two injections, but are not well tolerated by patients that may require months or years of injections to treat chronic eye diseases.

Fig 7: Typical drug concentration profile in the vitreous following an intravitreal injection

2.6 Diffusion and Convection

Diffusion: Molecular level transport of a species through another species as a result of a concentration gradient.

$$\text{Mass Flux of A via Diffusion} = \mathbf{J}_A = -\mathbf{D}_{AB} * [dC_A/db] - (\text{Fick's Law})$$

$$J_A = \text{flux of A due to diffusion} - M/L^2T$$

$$D_{AB} = \text{diffusivity of A in B (area/time)} - L^2/T$$

$$C_A = \text{concentration of A (moles or mass/vol)} - M/L^3$$

$$db \text{ is the differential length (length)} - L$$

Convection: Bulk flow which occurs under a pressure gradient or other imposed external force.

$$\text{Mass Flux of A via Convection} = C_A * U - (M/L^2T)$$

$$U = \text{volume average velocity of the fluid (length/time)} - L/T$$

$$\text{Total flux of A} = N_A = \text{Sum of diffusive flux of A} + \text{convective flux of A}$$

$$N_A = -(D_{AB}) * [dC_A/db] + C_A * U$$

$$(M/L^2T) = (L^2/T) * (M/L^4) + (M/L^3) * (L/T)$$

$$(M/L^2T) = (M/L^2T) + (M/L^2T) - (\text{Dimensionally homogeneous})$$

Peclet Number (Pe) is a non-dimensional parameter that relates advection and diffusion in the transport of species (or scalars) in a given system. If we describe advection and diffusion in terms of characteristic time scales, the peclet number is the ratio of these time scales,

$$Pe = \text{Diffusion Time scale } (T_D) / \text{Advection time scale } (T_C)$$

Preliminary Analysis (hand calculations):

Diffusion time scale:

$T_D \sim L^2/D$, where L is the characteristic length of the vitreous, and D is the diffusivity of methotrexate in vitreous.

$$Rabbit (T_D) \sim (0.78cm)^2/7*10^{-6}cm^2sec^{-1} \sim 24 \text{ hrs}$$

$$Human (T_D) \sim (1.03cm)^2/7*10^{-6}cm^2sec^{-1} \sim 40 \text{ hrs}$$

Convection time scale:

$T_C \sim L/U$, where L is the characteristic length of the vitreous, and U is the flow speed.

$$Rabbit (T_C) \sim 0.78cm/0.011cmhr^{-1} \sim 71 \text{ hrs}$$

$$Human (T_C) \sim 1.03cm/0.013733cmhr^{-1} \sim 75 \text{ hrs}$$

Therefore,

$$Pe_{Rabbit} \sim T_D/ T_U \sim 24/71 \sim 0.338028$$

$$Pe_{human} \sim T_D/ T_U \sim 40/75 \sim 0.5333$$

The Peclet number calculated for rabbit and human eyes in the vitreous was less than 1. This suggests that the diffusion is a dominant transport mechanism for methotrexate in the vitreous. Also, the diffusivity (D) of methotrexate in the vitreous is same for both rabbit and human eyes, it takes a longer time ($T_D \sim L^2/D$) for the drug to diffuse to retina and choroid in a human eye (40 hrs) as compared to a rabbit eye (24 hrs) because of the larger length L of the human eye. This implies that the residence time of the drug is greater in a human eye as compared to rabbit eye.

CHAPTER 3

Numerical Investigation of Kinetics of Methotrexate in a Human Eye

In this chapter, the geometry of the computational eye models, conservation equations and the boundary conditions used for the numerical calculations are explained. Furthermore, the kinetics of methotrexate in human eye following the standard I-C-M protocols is discussed in detail.

3.1 Methodology: Numerical Study

In this section, various model parameters and the solution methodology employed for evaluating the convective-diffusive transport of methotrexate within rabbit and human eyes are explained.

3.1.1 Model Development and Conservation Equations

The 3-dimensional model of the rabbit eye was adapted from our previous studies and a human eye model was constructed based on its physiological dimensions.^{26, 27, 30-34} The cross sectional view of the twelve-compartment eye models of rabbit eye and human eye are shown in Fig. 8. The 3D eye models consist of sclera (Fig 9A), cornea (Fig 9B), retina-choroid (Fig 9C), vitreous (assumed to be gelatinous) (Fig 9D), lens (Fig 9E), Schlemm's canal (Fig 9F), hyaloid membrane (Fig 9G), posterior chamber (Fig 9H), iris (Fig 9I), anterior chamber (Fig 9G), and a drug source at desired location (Fig 9K). The most significant differences between a rabbit eye and a human eye are the size of lens, volume of vitreous humor, and the flow dynamics inside posterior and anterior chambers. The volume of the vitreous humor in rabbit and human eyes is 1.5 ml and 4 ml, respectively.^{11, 31-33}

Fig 8: Cross sectional view of human and rabbit eye models. (A) Twelve compartments of the eye models (B) Dimensions of the eye models.

**Fig 9: Compartments of the eye models: (A) Sclera; (B) Cornea;
(C) Retina-Choroid and (D) Vitreous**

Fig 9: Compartments of the eye models: (E) Lens; (F) Schlemm's Canal;

(G) Hyaloid Membrane and (H) Posterior Chamber

**Fig 9: Compartments of the eye models: (I) Iris; (J) Anterior Chamber;
(K) Injection/Implant Site**

The unique physiological retinal permeability of any drug can be estimated numerically, if the exact initial location of the drug source (injection or implantation site) and the distribution of the drug are known from the *in vivo* studies. In this study, the retinal permeability of methotrexate was determined computationally by developing numerical eye models matching with the experimental conditions. The location and volume of the injection site, and the total mass of methotrexate used for the model calculations corresponded with the previously reported *in vivo* and clinical data,^{8, 11} which is shown in Table 1. Half eye models were constructed for both rabbit and human with the unique symmetry plane passing through the middle of the drug site and other eye compartments.

Table I. *In vivo* studies used for the estimation of retinal permeability of MTX

Model	Injection Location	Administered amount	Reference
Rabbit	3 mm posterior to Limbus	400 µg in 32 µL	Velez et al. ¹¹
Human	3 mm posterior to Limbus	400 µg in 100 µL	De Smet et al. ⁸

With the calculated retinal permeability value, kinetics of methotrexate was then analyzed in a human eye following an intravitreal injection and intravitreal implant. In the clinical studies, the methotrexate was intravitreally injected in to the human subjects at the level of *pars plana*. Thus, in this study, the drug source was modeled closer to the hyaloid membrane for simulating the kinetics of methotrexate in a human eye. Based on the data from previously published literature, an injection volume of 100 µL was chosen for simulating different injection doses, whereas the 8.5 mg implant was modeled for evaluating the therapeutic release rate of a 90-day intravitreal implant.^{7-9, 23, 24, 35} The drug

site was represented as a cylinder in our numerical models for both injection and implant (i.e., the injected drug was assumed to take the shape of a cylinder in the vitreous).

The distribution and elimination characteristics of any drug administered into the eye are significantly affected by both convection and diffusion. The diffusion of the drug in the eye is driven by the concentration gradient and the convection is due to the steady permeation of vitreous humor into the posterior segment of the eye. The flows of aqueous and vitreous humor were assumed to be steady and independent of drug concentration. The coupled convective-diffusive transport of methotrexate in the eye was evaluated by solving the nonlinear Navier-Stokes equations (Eqs. 1 and 2). The distribution of methotrexate was then calculated by coupling the species mass (methotrexate) conservation equation (Eq. 3) with the calculated flow field.³⁶

$$\rho \nabla \cdot (U) = 0 \quad (1)$$

$$\rho U \cdot \Delta U = -\nabla P + \mu \nabla^2 U \quad (2)$$

$$\frac{\partial C}{\partial t} + U \cdot \nabla C = D \nabla^2 C \quad (3)$$

where, P is pressure (g/cm-s² or μ Pa), U is velocity vector (cm/s), ρ and μ are the density (g/cm³) and viscosity (g/cm-s), respectively, of aqueous and vitreous humor, and C and D are the concentration (g/ml or μ M) and diffusivity (cm²/s) of the drug, respectively.

The initial and boundary conditions used for the model calculations are explained below. The ciliary process was modeled as a fluid source, which generates aqueous humor

at a constant flow rate of 2.2 $\mu\text{L}/\text{min}$ in a rabbit eye and 2.5 $\mu\text{L}/\text{min}$ in a human eye.^{27, 34}

The vitreous outflow was modeled as 0.1 $\mu\text{L}/\text{min}$ and 0.14 $\mu\text{L}/\text{min}$ for rabbit and human eyes, respectively.^{30, 34} The pressure at the outer surface of the sclera was set to zero gauge pressure and the upstream pressure was calculated based on the prescribed flow rate as boundary condition. The choroid was treated as a perfect sink and the drug passing through it was assumed to be cleared by the vast network of blood capillaries. So, the drug concentration at the outer surface of the retina-choroid was set to zero.^{26, 27} Also, a zero species gradient boundary condition was imposed at the outer surface of the Schlemm's canal, at all the symmetry surfaces, and also at surfaces that are assumed to be impermeable to the drug (iris, lens and cornea).

$$C = 0 \quad - \text{ at the outer surface of the retina-choroid}$$

$$\frac{\partial C}{\partial n} = 0 \quad - \text{ at the outer surface of the Schlemm's Canal lens, iris, cornea, and symmetrical surfaces}$$

Intravitreal injection of methotrexate with various doses was modeled by specifying a corresponding initial concentration of the drug throughout the cylindrical injection volume. In contrast, for an intravitreal implant with different release rates, an appropriate amount of drug was delivered with a constant flux at the outer surface of the drug source over a period of 90 days. Elsewhere in the model, the initial species concentration was set to zero for both injection and implant.

$$C_{t=0} = 8 \times 10^{-3} \text{ g/cm}^3 \text{ (for an injection dose of } 400\mu\text{g)}$$

$$D\left(\frac{\partial C}{\partial n}\right)_{t=0-90 \text{ days}} = 1.13568 \times 10^{-5} \text{ g/cm}^2\text{-day (for an implant loaded with } 400\mu\text{g)}$$

3.1.2 Model Parameters

The fluid properties of the aqueous and vitreous humor were nearly identical to that of water; hence, a viscosity of 6.9×10^{-3} g/cm-s and a density of 1 g/cm^3 were used for the computations. Since the diffusivity value of methotrexate was not available from published literature, the diffusivity of methotrexate for different compartments in the eye model was estimated (Eq. 4) from diffusion coefficients of ganciclovir that were previously published.³⁷ Using the Wilke-Chang correlation and assuming that the molar volume of each species at its normal boiling point is proportional to its molecular weight leads to the following expression:

$$\frac{D_{MTX}}{D_{GCV}} = \left(\frac{MW_{GCV}}{MW_{MTX}} \right)^{0.6} \quad (4)$$

where, D and MW are the diffusivity and molecular weight, respectively, of MTX and GCV. The diffusivity values of methotrexate were determined as $7 \times 10^{-6} \text{ cm}^2/\text{s}$ in the vitreous and $7.1 \times 10^{-6} \text{ cm}^2/\text{s}$ in posterior and anterior chambers.

The retinal permeability value of methotrexate was the only unknown parameter in the model and numerical calculations were conducted by varying its value from $1 \times 10^{-4} \text{ cm/s}$ to $1 \times 10^{-7} \text{ cm/s}$. The numerically obtained data was then compared with the *in vivo* and clinical data to determine the retinal permeability value of methotrexate for rabbit and human eyes. Using the calculated retinal permeability of methotrexate, numerical calculations were performed to analyze the kinetics of methotrexate in a human eye following the existing I-C-M protocols.^{7,9} The intravitreal injections of methotrexate were simulated in the human eye according to the standard protocol (De Smet et al.⁷) of twice-

weekly injections for four weeks (induction phase), followed by weekly consolidation injections for one month (consolidation phase), and followed by monthly injections for 9 months to 1 year (maintenance phase). The above protocol has been modified at Hadassah University hospital (Frenkel et al.⁹) such that the consolidation injections were given for two months instead of one month of the treatment period. The kinetics of methotrexate following the protocols used by De Smet et al.⁷ and Frenkel et al.⁹ were evaluated. Furthermore, the release rate of an intravitreal implant that would maintain therapeutic levels of methotrexate for three months in the vitreous of a human eye was estimated.

3.1.3 Solution Methodology

The human and rabbit eye models were meshed with 8 nodal hexahedral elements and are shown in the Figs10a and 10b, respectively. To avoid distortion of elements in the eye compartments, a total of 260565 elements were used. The skewness of the grid was less than 0.75 and aspect ratio was well below the acceptable level of 15. The Eqs. (1) and (2) were solved numerically by the Galerkin finite element method [FIDAP] involving nonlinear algebraic equations of the form,³⁶

$$M(\dot{V}) + K(U)V = F(U)$$

where, $K(U)$ is the global system matrix developed from the conservation of species transfer, M is the mass matrix, and F is the forcing function. Segregated solver was used for the spatial integration of the matrix equations. A second-order trapezoidal method, an implicit time integration scheme, was used.^{26, 27} The time step was variable and depended on the temporal variation of the species concentrations and their gradients. Convergence criterion for continuity and velocity residuals was kept at 10^{-6} , three orders of magnitude lower than the recommended value of 0.001.

Temporal integration scheme

FIDAP offers two general types of time integration schemes -- implicit and explicit (fixed and variable time step). An implicit second-order trapezoidal time integration scheme, with variable time increments, was used for our calculations. For our calculations, the maximum allowable increase in the time increment Δt (i.e is an upper bound for the ratio of the time step increment at time step $n + 1$ to the time step increment at time step n) for any one time step was set to 1.025. The first 3-4 time steps are computed using backward Euler with a fixed time increment. We have used the following parameters for our transient simulations:

$$T_{\text{start}} = 0$$

$$T_{\text{end}} = 180$$

$$\text{Maximum allowable increment (INCMAX)} = 1.025$$

$$\Delta T = 1/60$$

Spatial integration scheme

Segregated solver was used for the spatial integration of the matrix equations. Three variants of the segregated solver (pressure correction, pressure update and pressure projection version) are available in FIDAP. In this study, we have used pressure projection algorithm which solves directly for the pressure and performs a mass adjustment of the velocity field at the end of each iteration. This ensures that the velocity field satisfies the continuity equation at each iteration rather than only when convergence is achieved. The most important feature of the segregated solver is that it avoids the direct formation of a global system matrix. Instead, this matrix is decomposed into smaller sub-matrices each governing the nodal unknowns associated with only one conservation equation. These smaller sub-matrices are then solved in a sequential manner using either direct Gaussian elimination or conjugate gradient type schemes (iterative). In this study, the conjugate

residual iterative scheme was used for our calculations. The iterative methods begin with an initial guess and compute a sequence of approximate solutions that converge to the exact solution. The accuracy of the solution depends on the number of iterations performed.

Conjugate Residual (CR) Method - Algorithm

The CR algorithm can be viewed as a direct method that in the absence of round-off error gives the exact solution in N steps, where N is the number of unknowns, or, as an iterative procedure that gives an approximate solution with acceptable accuracy in far fewer steps. The basic conjugate gradient (CR) algorithm is as follows: consider the system of N linear equations.

$$AX = b \text{ (where } A \text{ is a symmetric matrix)}$$

Given an initial guess x_0 , a sequence of approximations to the solution x is generated as follows:

1. Choose x_0
2. Set $r_0 = b - Ax_0$
3. Set $P_0 = r_0$
4. For $i = 0, 1, 2, \dots$ until convergence do:

$$\alpha_i = (r_i, Ar_i) / (Ap_i, Ap_i)$$

$$x_{i+1} = x_i + \alpha_i p_i$$

$$r_{i+1} = r_i - \alpha_i Ap_i$$

$$b_i = (r_{i+1}, Ar_{i+1}) / (r_i, Ar_i)$$

$$p_{i+1} = r_{i+1} + b_i p_i$$

$$Ap_{i+1} = Ar_{i+1} + b_i Ap_i$$

Fig 10: (A) Computational grid - Human Eye

Fig 10: (B) Computational grid - Rabbit Eye

3.2 Results: Numerical Study

In this section, the retinal permeability value of methotrexate estimated for rabbit and human eyes was explained. The kinetics of methotrexate in a human eye following the standard I-C-M protocols (that are currently being used for the treatment of PIOL) is discussed in detail. The mean release rate of a 90-day methotrexate implant that needs to be achieved in the *in vitro* experiments for maintaining therapeutic levels in the vitreous is discussed in this thesis

3.2.1 Retinal Permeability and Half Life of Methotrexate in Rabbit and Human Eyes

The best fit data obtained between the simulated methotrexate vitreous concentrations and the experimentally determined concentrations published by Velez et al.¹¹ and De Smet et al.⁷ for rabbit and human eyes respectively are shown in Fig 11. For the selected material properties and boundary conditions, the calculated numerical concentrations agreed well with the *in vivo* and clinical data of methotrexate for rabbit and human eyes.^{8, 11} This validation provides support for the numerical methodology employed in the current study. The retinal permeability values of methotrexate obtained from the validation of numerical models with the experimental data (Fig 11), were 1.1×10^{-5} cm/s and 9.25×10^{-6} cm/s for rabbit and human eyes, respectively.

Fig 11: Comparison of the numerically calculated vitreous concentrations with the available *in vivo* and clinical data. (A) Numerical eye models were matched with the experimental conditions (injection location, volume and dose); (B) Comparison of simulated MTX vitreous concentrations and the experimentally determined concentrations published by Velez et al.¹¹ and De Smet et al.⁷ Exponential fit for the *in vivo* data of rabbit and human eyes were represented as grey dotted lines.

A linear relation on a semi-logarithmic plot (Fig 11) suggests a first order elimination process for methotrexate. The half life of methotrexate following an intravitreal injection was determined by using the slopes (K_{el} , defined as the fraction of the drug in the eye eliminated per unit time) of mean vitreous concentration curve with time. The half-life ($t_{1/2} = 0.693 / K_{el}$) of methotrexate in the vitreous was determined as 7.1 hr for a rabbit eye and 14.3 hr for a human eye. The difference between half-lives of methotrexate in rabbit and human eyes could be attributed to the differences in their retinal permeability values and volume of the vitreous humor as well as its perfusion which, in turn, affected the drug clearance.

3.2.2 Elimination of Methotrexate in Rabbit and Human Eyes Following an Intravitreal Injection

The percentage cumulative amount of methotrexate cleared through choroid and Schlemm's canal of rabbit and human eyes following an intravitreal injection is shown in the Fig 12. The total amount of methotrexate cleared through choroid following an injection was calculated to be 13.6% for a rabbit eye and 7.1% for a human eye. The remaining 86.4% and 92.9% of methotrexate was washed out by the aqueous flow through the Schlemm's canal for rabbit and human eyes, respectively. The above result suggests that a greater amount of methotrexate is cleared through choroid for a rabbit eye when compared to a human eye. This increase in clearance of methotrexate through choroid of rabbit eye, when compared to a human eye, can be attributed to its high retinal permeability value. Thus, the retinal permeability of a drug has significant effect on its elimination characteristics, and the higher the permeability, the greater the elimination will be through choroid. Distribution of MTX in rabbit and human eyes at various times is shown in Figs 13 a, b, c and d.

Fig 12: Elimination characteristics of MTX in human and rabbit eyes following intravitreal injection. Total amount of MTX cleared through the choroid and schlemm's canal is shown in the bar graph.

Fig 13A: Distribution of MTX at $t = 0.02$ hrs. (A1) Rabbit Eye and (A2) Human Eye

Fig 13B: Distribution of MTX at t = 3hrs. (B1) Rabbit Eye and (B2) Human Eye

Fig 13C: Distribution of MTX at t = 7hrs. (C1) Rabbit Eye and (C2) Human Eye

Fig 13D: Distribution of MTX at t = 24hrs. (D1) Rabbit Eye and (D2) Human Eye

Fig 13E: Distribution of MTX at t = 48hrs. (E1) Rabbit Eye and (E2) Human Eye

3.2.3 Methotrexate Concentration in Human Eye Following Standard

Induction–Consolidation–Maintenance (I-C-M) Protocols

The mean vitreous concentration of methotrexate in a human eye for the existing I-C-M regimens in which methotrexate was re-injected at different time intervals is shown in Fig 14. The treatment protocols used by De Smet et al.⁷ and Frankel et al.⁹ were simulated for 5 months of the treatment period and are shown in the Fig 14A and Fig 14B, respectively. The duration for which methotrexate concentration levels are less than 0.001 μM in the maintenance phase has been represented as dotted lines in Figs 14A and 14B. The methotrexate concentration profile is similar in the induction phase for both the protocols, as the dosing interval and injection dose (400 μg) used were identical in both cases.

From Fig 14A, it can be noticed that the methotrexate levels drop below 0.001 μM just after two months of the treatment following the protocol used by De Smet et al.⁷ Whereas, in the case of protocol used by Frenkel et al.⁹, the drop in methotrexate levels to 0.001 μM , occur well after three months from the start of the treatment (Fig 14B). The peak concentration of methotrexate achieved during all three phases of treatment following the dosing interval that was selected in the above discussed standard regimens was approximately 269 μM or 122 $\mu\text{g/mL}$. Given that the dosage above 0.1 μM is considered tumoricidal, it can be stated from the Figs. 14A and 14B that the therapeutic methotrexate levels are consistently achieved only in the induction phase of the treatment and are nearly maintained during the consolidation phase. The methotrexate levels drop below the therapeutic range during the maintenance phase, following the above mentioned protocols.

Fig 14A: Model simulated kinetics of MTX in a human eye following the Induction–Consolidation–Maintenance (I-C-M) protocol

used by De Smet et al.⁷

Fig 14B: Model simulated kinetics of MTX in a human eye following the Induction–Consolidation–Maintenance (I-C-M) protocol used by Frenkel et al.⁹

3.2.4 Kinetics of Methotrexate in Human Eye Following Different Doses of Intravitreal Injection

In order to determine the appropriate injection dose for the treatment of PIOL using the I-C-M protocols, kinetics of methotrexate following different injection doses needs evaluation. Hence, the variation of the mean vitreous concentration of methotrexate in a human eye with different doses of intravitreal injection was compared and is shown in Fig 15. The peak methotrexate concentration achieved in the vitreous following different injection doses, and the duration of time for which the methotrexate levels were within the therapeutic range, were calculated and are shown in Table 2. The average time for which methotrexate levels inside the vitreous were within therapeutic range following various injections doses was determined as 45 ± 4 hrs. The peak concentration of methotrexate in the vitreous following various injection doses is shown in Table 2. It is important to avoid high concentration of methotrexate that is toxic to normal tissues in the eye. From these results, one could not only select an appropriate injection dose, but also estimate the dosing interval for developing therapeutic protocols for PIOL using serial injections of methotrexate.

Fig 15: Mean vitreous concentration of MTX in a human eye following different doses of Intravitreal injection.

Table II. Duration time for which therapeutic MTX levels were achieved in the vitreous of human eye following different injection doses.

	Injected dose (μg)				
	200 μg	400 μg	600 μg	800 μg	1500 μg
Peak MTX Concentration achieved (μM)	132.7	264.8	396.5	529.4	982.3
Time (hr) for MTX Levels (> 1μM)	102	114	125	132	143
Time (hr) for MTX Levels (0.1 - 1μM)	46	49	47	44	41

3.2.5 Therapeutic Release Rate of a 90-day Sustained-Release Methotrexate Implant

The overall drug release profile can be controlled in the in vitro experiments by many factors including polymer microstructure, copolymer ratio, drug content and implant design. Using an effective sustained release device, therapeutic drug levels can be achieved in vitreous for 3-6 months, thus avoiding multiple surgeries. As a concluding part of this study, we have numerically calculated drug concentration in the eye based on a new generation implant having an optimal and a predetermined methotrexate-release rate that is within the therapeutic range. The temporal distribution of methotrexate inside the vitreous of a human eye following a 90-day implant with different release rates is shown in Fig 6. It can be noticed from Fig 6 that in order to achieve tumoricidal methotrexate levels in the vitreous for three months, a sustained-release implant with a mean release rate of 0.2 $\mu\text{g/day}$ - 2 $\mu\text{g/day}$ should be designed for therapeutic treatment of PIOL. For our calculations, we have assumed that drug will be released from the implant uniformly over time.

Fig 16: Mean vitreous concentration of MTX in a human eye following a 90-day controlled release implant with different release rates.

3.3 Discussion

PIOL provides a diagnostic and therapeutic challenge because of its non-specific ocular symptoms, diverse clinical picture, nonspecific presentation, and variable clinical course. The incidence of PIOL, which was previously a rare condition, has increased dramatically since 1970's. The response of tumors to the intravitreal injections of methotrexate is encouraging from the studies conducted by different research groups.^{8-10, 13, 35} Even though injection therapy gained widespread acceptance because of the complete remission of tumors, other alternatives need to be considered as intravitreal injection is associated with severe complications in some patients. At times during these treatments, the tolerance of the patients towards the injections was very poor. For such patients, the treatment period was reduced to two months; however, with an uncertainty of tumor recurrence.⁹ Hence, in the present study, we have numerically analyzed the kinetics of methotrexate in a human eye following the standard I-C-M injection protocols and investigated the scope for reducing the number of injections during the treatment.

The permeability of methotrexate across the retina has a significant impact on its clearance from the vitreous. It is a cumbersome process to measure the permeability of retinal tissues to various drugs through *in vivo* experiments. Thus, in this study, we have numerically calculated and determined the retinal permeability of methotrexate for albino rabbit and human eyes (Fig 11) by matching the calculated vitreous concentrations with the *in vivo* data (mean or volume averaged methotrexate concentration in entire vitreous) and clinical data (concentration of methotrexate in the extracted sample) that has been previously published.^{8, 11} The calculated retinal permeability of methotrexate in a human eye (pigmented mammal) was lower than that in the albino rabbit eye. This observation is consistent with the previous study, in which the retinal elimination rate constant

(normalized for retinal surface area, distribution volume and anatomic volume) of ganciclovir was slightly greater in a rabbit eye (0.017 cm²hr⁻¹) as compared to the human eye (0.015 cm²hr⁻¹), thus indicating a higher retinal permeability in rabbits.³⁸ However, in this study, the binding of methotrexate to ocular tissues, its metabolism and active transport process have not been incorporated in the numerical method. All the above mentioned factors could affect the retinal permeability of methotrexate and in turn affects the methotrexate distribution in the eye.^{39, 40}

Previous studies have investigated the inward and outward retinal permeability and attributed an active efflux mechanism for higher outward permeability.^{30, 41-43} This higher value of the permeability in the outward direction is because of the active pumping by the retinal pigment epithelium, which is known to pump water from retinal space in to the choroidal circulation. Since the retinal permeability is iterated and calculated to match the measured concentration data, the reaction kinetics for binding, association, dissociation, and other active transport are, in a way, indirectly included in the retinal permeability value. In other words, the value of retinal permeability is a lumped or bulk parameter that includes the effect of reaction kinetics and active transport in an indirect manner.

Most of the drugs that are intravitreally administered will be eliminated through anterior and posterior routes. The clearance of methotrexate in rabbit and human eyes following an intravitreal injection was investigated in this study (Fig 12). It was observed that because of high retinal permeability of methotrexate in the rabbit eye when compared to that of a human eye, there is greater clearance of methotrexate through choroid in a rabbit eye as opposed to that in a human eye. Previous numerical studies have shown that increasing the retinal permeability increases the rate of clearance of drug from the eye, thus

resulting in a lower half life.^{26, 32} Also, it is apparent that if the drug source was modeled close to the retina or at the center of the eye, the amount of methotrexate washed away by aqueous humor will be reduced.

Half-lives of most of the drugs that are injected in to vitreous cavity are predominantly low because of the continuous clearance of the drug across the blood eye barriers, aqueous humor turn over and uveal blood flow. Since the half life of methotrexate is of the order of only a few hours, frequent injections are required to maintain cytotoxic levels, which are often associated with ocular complications such as vitreous hemorrhage, endophthalmitis, and retinal detachment. Initial clinical experiments have shown that tumoricidal methotrexate levels can be maintained in the vitreous for up to 3-5 days following an intravitreal injection (400 µg), suggesting a rapid elimination of the drug.⁸ Hence, in order to maintain tumoricidal methotrexate levels consistently for a longer duration (2-3 months), repeated administrations are necessary. Based on this result, I-C-M protocols have been developed such that twice-weekly, weekly, and monthly injections were given as induction, consolidation and maintenance phases, respectively. Different research groups have used the I-C-M injection protocols with some changes in the dosing intervals.^{7, 9, 10} In a study conducted on an Asian patient¹⁰, twice-weekly injections of methotrexate were given for three weeks as compared to the standard four weeks of the induction phase.

In the present study, we have simulated the kinetics of methotrexate in a human eye following the protocols used by De Smet et al.⁷ and Frenkel et al.⁹ (Figs 14A and 14B), as it is not feasible to experimentally measure the concentration levels of methotrexate at different time points during the treatment period. Following the above mentioned I-C-M

protocols with an injection dose of 400 µg, cytotoxic levels of methotrexate were not achieved consistently in the vitreous for part of the consolidation and maintenance phases of the treatment. We have calculated the mean vitreous concentration of methotrexate in a human eye for various injection doses not only to analyze the scope of using different injection doses for I-C-M protocols but also to reduce the number of injections during the treatment. It can be observed (Fig 15) that tumoricidal methotrexate levels ($> 1 \mu\text{M}$) can still be achieved in the induction phase by using a lower injection dose of 200 µg instead of 400 µg. By using an injection dose of 200 µg in the induction phase of the treatment, the peak concentration of methotrexate can be reduced by 50% as compared to an injection dose of 400 µg.

It is evident from Fig 15 that even by increasing the injection dose ($> 400 \mu\text{g}$), it might not be possible to reduce the number of injections significantly during the treatment, as the methotrexate is rapidly eliminated from the vitreous. By increasing the injection dose, higher methotrexate levels ($> 0.1 \mu\text{M}$) can be achieved for a few additional days when compared to the lower injection doses, but it could lead to higher toxicity to normal retinal tissues. The other alternative is to vary the dosing interval so that cytotoxic methotrexate levels are achieved during the treatment period with minimum number of injections. It can be noticed from Figs 14A and 14B that the number of injections needed for the treatment could be significantly reduced by giving weekly injections in the induction phase instead of twice-weekly injections. From our numerical calculations (Figs 14A and 14B), we have evaluated that the tumoricidal levels of methotrexate are reasonably achieved in the consolidation phase following weekly injections (400 µg). Thus, the possibility of having induction and consolidation phases with the weekly injections (with a dose $\geq 400 \mu\text{g}$) for the first 2-3 months of the treatment period, can be considered in the future experiments.

The results of this study when used in combination with the toxicological studies could provide more information about the appropriate injection dose and the dosing interval that needs to be developed for the therapeutic treatment of PIOL.

Considering the tolerance of patient and the surgical procedure involved in injection therapy, development of new treatment modality is necessary. Various sustained-release devices have been investigated to achieve therapeutic drug levels in the eye while avoiding toxic or sub-therapeutic levels and some of these studies have reached clinical phase. In a study conducted by Whitcup et al.⁴⁴ sustained-release methotrexate implants were developed *in vitro* using polyvinyl alcohol for the treatment of PIOL. However, it was reported that the implants were designed to maintain therapeutic levels only for two weeks to one month. Considering that the cytotoxic methotrexate levels need to be achieved for at least two months (to induce some remission of tumor), there is a need to develop better sustained-release devices to deliver prolonged therapeutic methotrexate levels for the treatment of PIOL. In the current study, we have numerically simulated the kinetics of a 90-day controlled release methotrexate implant and determined its therapeutic release rate, in order to achieve tumoricidal levels in a human eye for three months. This study is based on a release rate of a newer generation implant for which the first and second bursts are minimal.

In summary, we have numerically calculated the retinal permeability value of methotrexate in human and rabbit eyes by developing numerical eye models and matching them with the experimental data from the previous studies. We then investigated the kinetics of methotrexate in a human eye following the standard I-C-M protocols and analyzed the scope of reducing the number of injections during the treatment period. Also,

we have considered an alternative treatment technique, intravitreal methotrexate implant, and evaluated its therapeutic release rate in order to achieve prolonged cytotoxic levels of methotrexate in the vitreous, thus avoiding toxic or sub-therapeutic methotrexate levels. By using the results of this study, one could select a dosing interval for serial injections of methotrexate or establish a treatment schedule using controlled release intravitreal implant for the treatment of PIOL.

CHAPTER 4

Toxicity Assessment of a Biodegradable Implant Loaded with Methotrexate in Normal Rabbit Eye

In this chapter, the design and fabrication procedure of the biodegradable implants loaded with methotrexate is explained. The implantation procedure employed in our study animals, and the clinical and pathologic studies performed to evaluate the safety of these devices are explained. Furthermore, the histopathological findings of the *in vivo* study are discussed in detail.

4.1 Material and Methods: *In Vivo* Study

In this section, the materials used for the fabrication of biodegradable implant and the implantation procedure are explained.

4.1.1 Design and Device Fabrication

The process for the fabrication of a biodegradable-polymeric implant to potentially deliver methotrexate is based on a micromolding technique from high-aspect-ratio poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning, Midland, MI, USA).⁴⁵ Firstly, the cycloolefin copolymer (COC) was laser drilled and the steel needles were fitted into the holes to create the master structure as shown in Fig 17. Secondly, the micro-needle master structure was coated with PDMS, which was subsequently peeled off to make an inverse mold. This PDMS mold was used to fabricate polymeric micro-needle implants by using the solvent cast method. The design of the micro-needle implant was adapted from our previous study and is shown in Fig 18A.⁴⁶ The 9-needle biodegradable methotrexate implant weighed approximately 8 mg and was approximately 2 mm in length, 2 mm in

width, and 2.3,mm in height as shown in Fig 18B. The tips of the micro-needles were beveled at an angle of about 30° to facilitate their intended penetration through partial thickness sclera and into the underlying uvea.

Poly (D,L-lactide) (PLA) with a high weight-average molecular weight of 90,000 (PLA-90,000) and a low weight-average molecular weight of 6,500 (PLA – 6,500) were purchased from Lactel Biodegradable polymers (Alabama, USA). To prepare the sustained release methotrexate implants, the solution of PLA-90,000/PLA-6,500 (blend ratio; 80/20) and 10% wt of methotrexate (Sigma-Aldrich, St. Louis, MO, USA) dissolved in N-Methylpyrrolidone (HPLC grade) solvent, was cast in the PDMS mold. Negative pressure was applied to PDMS mold to load the solution into the needle part and then the implant was dried for three days. To increase the mechanical strength of the implant, the solution in the PDMS mold was heated to glass transition temperature. The placebo implants (polymers without drug) were prepared in a similar fashion. The implants were all rigid at room temperatures and at lower temperatures.

Figure 17: The fabrication procedure used for making the biodegradable polymeric MTX implant based on micromolding technique from high-aspect-ratio poly(dimethylsiloxane) (PDMS); (Source: Park et al., 2006)

B

Figure 18: Biodegradable polymeric MTX implant used for this study (A) Design of the implant (Source: Park et al., 2006); (B) Actual MTX implant used in the *in vivo* experiments.

4.1.2 Device Implantation

All animals were treated in accordance with the IACUC protocol (University of Cincinnati) for the use of animals in the current research. In this study, three New Zealand white rabbits weighing 2 – 3 Kg each were used for *in vivo* study. An implant containing methotrexate was placed surgically in a deep lamellar scleral pocket in the right eye, and a placebo device was placed in a comparable deep lamellar scleral pocket in the left eye of each rabbit using the surgical method described below.

Prior to anesthesia administration, both pupils of the animals were dilated using two drops of phenylephrine hydrochloride 2.5% and two drops of tropicamide 1%. The rabbits were anesthetized with a mixture of xylazine hydrochloride (5 mg/Kg) and ketamine hydrochloride (35 mg/Kg). Following anesthesia, a 24-gauge angiocatheter was inserted into the marginal ear vein to facilitate antibiotic administration; the pulse, temperature, and respiration rate were monitored to ensure sufficient anesthesia and satisfactory vital signs. The procedure started with the aseptic preparation and sterile draping of both eyes. The procedure was essentially the same in both eyes starting with the right eye. An eyelid speculum was inserted between the eyelids to expose the eye. A limited conjunctival peritomy was performed in the superotemporal quadrant, and the subconjunctival connective tissues were dissected down to bare sclera in that quadrant as shown in Fig 19A. Using a surgical blade, a square shaped fornix based deep lamellar scleral flap measuring 3.5 by 3.5 mm was created in the superotemporal quadrant of the eye. The center of the lamellar scleral flap was located approximately 3 to 3.5 mm from the corneoscleral limbus. The inner lamellar sclera (where the outer lamellar scleral flap was elevated) was thin enough to allow an examination of the dark underlying uveal tissue.

The fabricated implant was placed inside the flap with the needles firmly pressed against the inner lamellar sclera as shown in Fig 19B. The scleral flap was closed immediately with 8-0 monofilament nylon sutures. The conjunctival peritomy was then closed using 7-0 absorbable sutures as shown in Fig 19C. The micro-needles were expected to penetrate the inner scleral lamellae in the scleral pocket and the underlying uveal tissue of the ciliary body and project into the peripheral vitreous.

After the procedure, the animals received post anesthesia care and were returned to their cages after satisfactory recovery was documented. Besides their usual care, they received topical antibiotic drops in both eyes three times a day for one week. After surgical hemostasis, no external bleeding or vitreous hemorrhage in any eye was observed during or after the surgical procedure. Four weeks after the implantation, the animals were killed by an overdose of intravenous sodium pentobarbital and the eyes were enucleated. The enucleated globes were labeled and stored at -80⁰C in 10% neutral buffered formalin for later sectioning and histopathologic examination.

4.1.3 Histopathology Study

The globes were fixed for at least 12 hours and subsequently sectioned so as to display as much of the implant site as possible but also including the pupil and optic nerve (P.O. section). The sectioned portion of the specimen was then processed overnight to remove most of the water from the tissue and allow replacement by paraffin. The paraffin mechanically stabilizes the tissue, making possible the cutting of thin sections (4-6 µm thick) for tissue staining with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS).

Figure 19: Implantation procedure employed in the animal experiments (A) Scleral flap made in the superotemporal quadrant of the eye (B) Device implantation and (C) Suturing the scleral flap and conjunctival peritomy.

Hematoxylin-eosin (H&E) staining method is a standard staining method used in histology. It gives an overview of the structure of the tissue, enabling differentiation of the structures being examined as normal, inflamed or degeneratively changed, or pathological. It is often possible to make a diagnosis based on the results of H&E staining, or it can be determined which special staining techniques are still necessary for subtyping. It's actually a combination of two dyes: the basic dye hematoxylin, and the alcohol-based synthetic material, eosin. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. Eosin is strongly absorbed by red blood cells, coloring them bright red. Some structures do not stain well using H&E and they need to need to be stained by other techniques such as PAS stain or some silver stains, if they have to be well visible.

Periodic acid-Schiff (PAS) is a staining method used to detect glycogen in tissues. The reaction of periodic acid selectively oxidizes the glucose residues, creates aldehydes that react with the Schiff reagent and creates a purple-magenta color. It is used for the detection of glycogen in tissues such as liver, cardiac and skeletal muscle on formalin-fixed, paraffin-embedded tissue sections, and may be used for frozen sections as well. The glycogen, mucin, and fungi will be stained purple and the nuclei will be stained blue.

In this study, five microslides were made of each globe, three stained with H&E and two with PAS. These slides were evaluated by two blinded, independent ophthalmic pathologists.

4.2 Results: Histopathological Findings

The implant was intended to be placed in the deep lamellar scleral pocket with its micro

needles penetrating the remaining inner lamellar sclera and underlying ciliary body stroma and ciliary epithelial layers and be long enough to project into the peripheral vitreous directed toward the interior of the eye. The needles themselves (rather than the body of the implant) were then supposed to deliver the contained medication directly into the vitreous. However, during the surgery an unanticipated softening of the micro-needle implant occurred when the implant came in contact with the scleral tissue that was at body temperature (higher) compared to the refrigerator or storage temperature (lower) of the micro-needle implant. The micro-needles were not rigid as intended (due to the heat induced implant softening) and did not penetrate any eye wall structures. Consequently, the implant was not placed at intended position but was simply placed in the intrascleral pocket. No other intra-operative or post-operative unexpected findings and complications were present in either of the studied eyes.

The pertinent histopathological findings were the presence of limited granulomatous inflammation in the intrascleral pocket, and residual implant material in all three right eyes examined. In contrast, all three left eyes examined presented an empty intrascleral pocket and no inflammation related to the implant. The left eye of rabbit # 1 presented limited scar tissue around the intrascleral pocket, and the left eye of rabbit # 3 exhibited rare foreign body giant cells surrounding one remaining suture. The inner sclera appeared intact in all cases.

Neither one of the eyes that received methotrexate implants or the eyes that received placebo implants showed any evidence of toxicity caused by the polymeric implant, the methotrexate or by both combined. Histopathological examination showed no appreciable abnormalities of the cornea, anterior chamber, iris, lens, ciliary body or retina in any eye. Discrete differences were apparent between the methotrexate containing

implants versus the placebo implants. Examination of intrascleral pockets (Fig 20A) showed no traces of implant material in eyes that received placebo implants (without methotrexate in left eyes), whereas for the eyes that received medicated implants (with methotrexate in right eyes), residues of the implant were found inside the intrascleral pockets (Fig 20B). The arrows in Fig 20B indicate the locations of the residual implant material.

An unrelated finding was a limited foreign body reaction close to the suture material (indicated by arrows) that was observed in the left eye (treated with placebo) of animal # 3 (Fig 20C). No ocular inflammation was evident around the implantation site in the right eyes that received the methotrexate implant. However, limited granulomatous (foreign body giant cells) inflammation was observed in the right eyes around the areas having residual implant (indicated by arrows) in all the rabbits (Fig 20D). There was no histopathological evidence that the implant micro-needles penetrated the inner lamellar sclera. The photomicrographs of the empty intrascleral pocket in the left eye (H&E-20X) and the intrascleral pocket containing implant residue in the right eye (H&E-20X) of animal # 2 are shown in Fig 20A and Fig 20B, respectively. Fig 20C shows the foreign body giant cell reaction to suture material observed in the left eye of animal # 3 (H&E-20X). The foreign body inflammation observed in the right eye of animal # 1 (H&E-40X) is shown in Fig 20D.

Figure 20: Histopathologic examination of rabbit eyes four weeks after the implantation. Photomicrographs of (A) Empty intrascleral pocket in the left eye of animal 2 (H&E-20X), (B) Intrascleral pocket containing implant residue in the right eye of animal 2 (H&E-20X)m (C) Foreign body giant cell reaction to suture material in the left eye of animal 3 (H&E-20X) and (D) Foreign body inflammation observed in the right eye of animal 3 (H&E-20X).

4.3 Discussion

The incidence of PIOL, which was previously a rare condition, appears to have increased substantially since the 1970's. Although intravitreal injection therapy using methotrexate (off-label use) is now being used to treat many eyes with PIOL, this method of treatment suffers from uncertainty as to the duration of a satisfactory tumoricidal concentration of drug in the eye, and the necessity of performing multiple intravitreal injections to successfully eradicate intraocular disease. A well-tolerated non-toxic slow release device that delivers prolonged therapeutic methotrexate levels and avoids frequent intravitreal injections would represent a major advance in patient care. In this brief technical report, we describe our experience with a biodegradable micro-needle methotrexate implant in normal rabbit eyes as a possible treatment technique for PIOL.

Sustained-release devices with micro-needles have shown promising results in the field of transdermal drug.⁴⁷ Guided by the success of micro-needles in transdermal drug delivery, we have fabricated a biodegradable micro-needle implant to deliver prolonged therapeutic methotrexate levels for the treatment of PIOL. In our previous studies, we investigated the effect of 2-methoxyestradiol and methotrexate on antiproliferation, apoptosis, and cell cycling in human lymphoma cells and observed that both 2-methoxyestradiol and methotrexate are suitable drugs for treatment of PIOL in humans.⁴⁸ Also, we have developed a biodegradable micro-needle implant (8.5 mg) loaded with 2-Methoxyestradiol (10%, 25% and 40% wt/wt.). For 10% 2-Methoxyestradiol-loaded implants, we observed a tri-phasic drug-release profile in the *in vitro* experiments following the release characteristics of the polymer, due to the diffusion, and swelling, and disintegration of the polymeric micro-needle. The 10% wt 2-methoxyestradiol implants

released the drug completely within four weeks, whereas the 25% wt 2-methoxyestradiol implants maintained therapeutic levels ($0.1\mu\text{M}$ - $1\mu\text{M}$) for 90 days.

For the current *in vivo* study, we have used similar polymer combination (blend of high and low molecular poly(D,L-lactide) used in our previous study for preparing implants loaded with 2-Methoxyestradiol) for fabricating the micro-needle implants loaded with methotrexate (10% wt/wt) and investigated the safety of this implant in normal rabbit eyes. We anticipate that the micro-needle methotrexate implant used in this study will release the drug over eight weeks (similar to the polymer degradation observed for 2-Methoxyestradiol implants as discussed above). As stated before, the implants were intended to be placed in the deep lamellar scleral pocket with its micro needles penetrating the remaining inner lamellar sclera and underlying ciliary body stroma and ciliary epithelial layers. However, the implant has softened abruptly during the implantation. Consequently, the micro-needles were not rigid and did not penetrate any eye wall structures, and the implant was simply placed in the intrascleral pocket. This unintended implant placement (intrascleral pocket) was unlikely to yield any significant drug delivery to the vitreous and retina (i.e., this design proved to be a complete practical failure). The micro-needle implants loaded with methotrexate did not perform mechanically in the intended way.

The histopathology results showed that the softened implants (without and with the medication) placed in the intrascleral space (with none of its needles penetrating any eye wall structures) did not cause any obvious major toxicity to the eye. However, in the right eyes of all the rabbits (treated with methotrexate), limited granulomatous (foreign body giant cells) inflammation was observed around residual implant material. We speculate that this limited inflammatory reaction is due to some alteration of the drug or polymer related

to drug-polymer interaction as no inflammation was evident inside the intrascleral pocket of the left eyes treated with placebo implants. Since methotrexate is a hydrophilic (lipophobic) drug, its compatibility with biodegradable polymers is a complex issue. It is possible that a hydrophobic (lipophilic) drug such as 2-methoxyestradiol will not exhibit similar drug-polymer interaction. The drug-release characteristics of the softened implant and the clearance of methotrexate from the eye were not evaluated in this study.

Most of the chemotherapeutic drugs injected into the vitreous generally have a very short half-life (less than a week) indicative of rapid elimination from the eye. Repeated administrations of intravitreal drugs are usually necessary to maintain prolonged tumoricidal levels in the vitreous. Keeping this scenario in mind, the determination of therapeutic release rate of this biodegradable methotrexate implant and efficacy data for the treatment of PIOL would be needed to prove that implants have a therapeutic and sustained drug-release capability. Furthermore, because drug release implants usually take a few days to achieve therapeutic dose, one can speculate that a sustained release methotrexate implant coupled with a concurrent initial intravitreal injection of drug may be a safe, effective and alternative treatment for eyes with PIOL.

CHAPTER 5

Conclusions and Future Work

In this chapter, the key findings from the numerical study are summarized followed by the results of the *in vivo* study.

5.1 Numerical Study

The RP values of MTX, obtained from the validation of numerical models with the experimental data, were 1.1×10^{-5} cm/s and 9.25×10^{-6} cm/s for rabbit and human eyes, respectively. The half-life ($t_{1/2} = 0.693 / K_{el}$) of MTX in the vitreous was determined as 7.1 hr for a rabbit eye and 14.3 hr for a human eye. The total amount of MTX cleared through choroid following an injection was calculated to be 13.6% for a rabbit eye and 7.1% for a human eye. The remaining 86.4% and 92.9% of MTX was washed out by the aqueous flow through the Schlemm's canal for rabbit and human eyes, respectively.

It was observed that following the existing I-C-M protocols with an injection dose of 400 µg, cytotoxic levels of MTX were not achieved consistently in the vitreous for part of the consolidation and maintenance phases of the treatment. In order to achieve tumoricidal MTX levels in the vitreous for 3 months, a sustained release implant with a mean release rate of 0.2 µg/day - 2 µg/day should be designed for therapeutic treatment of PIOL. By making use of the results of this study, one could select a dosing interval for specific injection dose or a treatment schedule can be established by using an intravitreal implant for optimal treatment of PIOL. In the future work, the binding of MTX to ocular tissues, its metabolism and active transport process need to be incorporated in the numerical method. All the above mentioned factors could affect the RP of MTX and in

turn affects the MTX distribution in the eye.

5.2 *In Vivo* Study

In this study we have investigated the use of a biodegradable micro-needle implant loaded with methotrexate as a possible treatment technique for PIOL. The implant was intended to be placed in the deep lamellar scleral pocket with its micro-needles penetrating the remaining inner lamellar sclera and underlying ciliary body stroma and ciliary epithelial layers and be long enough to project into the peripheral vitreous directed toward the interior of the eye as shown in Figure 21.

Figure 21: Intended penetration of the biodegradable micro-needle implant.

Unfortunately, the implant has softened abruptly during the implantation procedure when they came in contact with the scleral tissue that was at body temperature of the rabbit. The micro-needles were therefore not rigid and did not penetrate any eye wall structures, and the implant was simply placed in the intrascleral pocket. This implant placement (unintended) was unlikely to yield any significant drug delivery to the vitreous and retina (i.e., this design proved to be a complete practical failure). The micro-needle implants loaded with methotrexate did not perform mechanically in the intended way. The

histopathology results showed softened implants (without and with the medication) placed in the intrascleral space (with none of its needles penetrating any eye wall structures) did not cause any obvious major toxicity to the eye. The drug-release characteristics of the softened implant and the clearance of methotrexate from the eye were not evaluated in this study.

For optimal treatment of PIOL, therapeutic levels of methotrexate need to be maintained in the vitreous for a prolonged period while avoiding toxic and sub-therapeutic levels. It is apparent that a device placed entirely in the vitreous (or at least with its drug containing portion projecting into the vitreous) will deliver higher therapeutic levels of drug to the vitreous than any intrascleral or supraciliary implant. Since the micro-needles of the bio-degradable implant did not perform as expected because of the heat induced softening, our group is currently considering alternative implant designs and fabrication procedures which not only offer improved mechanical strength (rigidity) but also allow us to place the implant directly in the vitreous via a full thickness eye wall microincision. The design we select for our next generation of implants will depend on the intended position of placement of the implant in the eye and the allowable parameters of implant size. The best implant would be one that is small, easy to fabricate, stable at a range of temperatures, is easy to implant (with limited direct and secondary traumatic effects on the eye), delivers therapeutic levels of contained drug at a uniform rate throughout its life span, and does not cause any normal tissue toxicity.

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