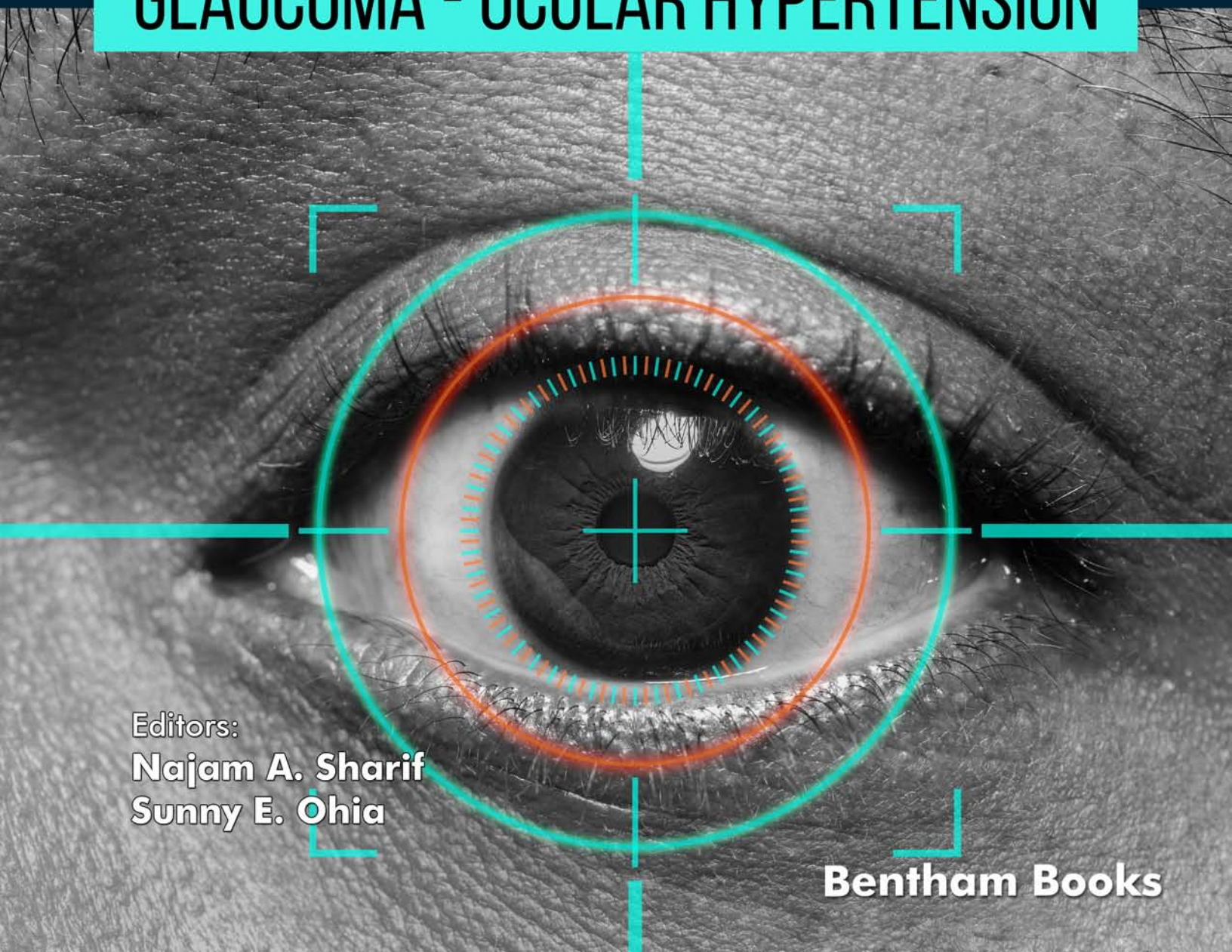
RESEARCH PROTOCOLS FOR OPHTHALMIC DISEASE MECHANISMS AND THERAPEUTICS:

GLAUCOMA - OCULAR HYPERTENSION



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Research Protocols for Ophthalmic Disease Mechanisms and Therapeutics: Glaucoma - Ocular Hypertension

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DEDICATION

This book is dedicated to God, my father (may his soul rest in peace, Amen), my mother, my siblings, my wife and children with whose blessings and support everything in my life has been rendered possible. *Najam A. Sharif*

Dedicated to my wife, Dr. Ekanem Ohia, and my children, Dr. Odochi Nwoko and Uchechukwu Ohia, Esq. *Sunny E. Ohia*

PREFACE

We are grateful for the opportunity to compile and edit this book, composed of two volumes, on materials and methods for conducting experiments to study the many aspects of the disease, ocular hypertension (OHT) and glaucoma, a constellation of potentially blinding diseases that afflict millions of patients worldwide. Additionally, it was important to guide readers about the drug / device discovery process and the basic aspects of drug development to treat the afore-mentioned eye diseases. Thus, salient features of Investigational New Drug (IND)-enabling studies for new compounds and devices are presented to underscore the ultimate purpose of the ocular research that is conducted in academia and industry, namely, to unearth mechanisms of ocular hypertensive disease and then find treatment options to mitigate and retard the disease process(es).

We have been conducting and supervising basic and applied ocular research for advancing knowledge about the pathogenesis of eye diseases, and drug discovery and development for more than three decades. Working in the pharmaceutical and academic arenas, respectively, and frequently guiding and mentoring junior staff, we realized there was a deficiency of a single source of key methods utilized for performing ocular pharmacology, disease mechanisms and therapeutics discovery research. To capture existing procedures and rapidly emerging techniques and technologies, we decided to develop this book. This project would not have been possible without the kind and generous help and support of our family members, Bentham Science Publishers and our many colleagues who have contributed to this endeavor. We have deliberately focused on OHT, which develops from elevated intraocular pressure (IOP) in the anterior chamber of the eye (ANCe) as a case study, since lowering and controlling IOP by pharmaceuticals, aqueous humor drainage devices and ocular surgery are the only currently approved medical treatments for OHT and for many forms of glaucoma. The topic of glaucomatous optic neuropathy (GON), which has several risk factors of which OHT is one, and that encompasses the many factors and events occurring at the optic nerve head, retina, optic nerve and the brain leading to potential blindness, will be covered in a follow-up book.

Protocols described in the various sections and chapters in the current two volumes cover a wide range of techniques that are currently being used in studying the anatomy, morphology, pathology, biochemistry, physiology, pharmacology, and molecular biology of the cells/tissues within the anterior chamber of the eye (ANCe). In Volume 1, Section 1, beginning with a brief review of the basic anatomy and physiology of the eye (Chapter 1), a treatise on research and development of drugs, their sources and screening paradigms *in vitro* and *in vivo* are described in Chapters 2 and 3. Since the majority of drugs used to treat elevated IOP engage with membranous and /or cytoplasmic receptors and/or enzymes, it is important to determine whether the tissues/cells contain the requisite drug targets. This is accomplished using specific protocols and a range of techniques (reverse transcriptase polymerase chain reaction [RT-PCR]; receptor binding; autoradiography and immunohistochemistry) that are described in Section 2 (Chapters 4-7). Once cellular binding site for target receptor/enzyme verification has been accomplished, it is necessary to demonstrate the functional activity associated with the target protein through drug-receptor engagement using well characterized reference compounds in suitable cell-based assays. Additional relevant cell types are also needed to potentially define off-target effects of test compounds and/or to find ocular cells that may contain the drug receptor/enzyme/transporter protein(s). This necessitates isolation, propagation and utility of cells from appropriate ANCe uveal tissues (*e.g.* cells from the ciliary muscle, ciliary processes, trabecular meshwork, Schlemm's canal) and other cells derived from the sclera, corneal and conjunctival tissues.

Suitable protocols needed for such endeavors are described in Section 3 (Chapters 8-13).

Cell- and tissue-based assays to permit interrogation of receptor/enzyme activity for compounds of interest are crucial in the quest to find new drugs for treating elevated IOP, and these are described in Section 4 (Chapters 14-24). Drug discovery screening, whether conducted manually or using automated high-through-screening platforms, requires the use of a broad range of biochemical and pharmacological techniques and technologies. Agonist or antagonist activity of reference and test compounds can be determined in isolated cells or tissues using a variety of functional parameters such as quantifying second messengers, cell impedance, cell volume changes, tissue contraction/relaxation, release of transmitters, cellular ionic changes, etc. The concentration-response data, including receptor/enzyme affinity, compound potency and intrinsic activity, can then help rank order compounds and find suitable hits for further optimization by the medicinal chemists. Once all the *in vitro* studies have been completed and suitable compounds selected to progress for testing in normal animals for ocular safety and tolerability, and then for efficacy, the more labor-intensive and expensive portion of drug/device discovery and development begins.

Although the *in vitro* pharmacological characterization of drug targets and test compounds is important, ultimately appropriate response(s) in animal models of disease dictates the final selection of a suitable lead compound, and back-ups, that can be further characterized via additional IND-enabling studies. These aspects are the subject matter covered in Volume 2 of this book. In the case of glaucoma/OHT, compounds should be safe (not causing eye irritation or other systemic/ central nervous system adverse effects) and be able to significantly lower and control IOP. Thus, Volume 2, Section 5 (Chapters 25-36) provides numerous protocols for such *in vivo* studies in various mammalian species (rodent, rabbit, dog, monkey) using induced methods to raise IOP in various animals. Since the aim of the IOP-reducing drugs for treating OHT/glaucoma is to promote AQH outflow from the ANCe and/or to reduce AQH production, a specific protocol dealing with AQH drainage devices (Chapter 36) is also provided. In the modern age the use of genetic medicine has come to fruition. Along the way, the discovery of naturally occurring mouse models of glaucoma and development of new animal models of OHT using genetic engineering has also been accomplished, and these are described in Section 6 (Chapters 37-39).

Once compound ocular safety and tolerability has been established, and some relative efficacy in a few models of OHT demonstrated, the lead compound and any back-up compounds can now be progressed to a stage where an ultimate formulation is selected for all remaining studies. Typically, a safe and comfortable drug formulation suitable for topical ocular dosing is developed and appropriate pharmacokinetic, pharmacodynamic and toxicological studies performed on the lead compound. Such investigations can be performed using protocols described in Section 7 (Chapters 40-42). The results from the *in vitro* and *in vivo* studies performed for the IND-ready compound(s) or device(s) using validated protocols and standard operating procedures (SOPs) using Good Laboratory Practices (GLP) and/or Good Manufacturing Practices (GMP) can then be assembled into dossiers for submission to the regulatory authority under an IND application. These and other key regulatory considerations need to be addressed through appropriate communications and interactions with the health agency and are out of scope for the current book.

Finally, we surmised that in this technologically advanced age we should provide the modern researchers in ocular physiology and pharmacology new methods to help advance basic and applied eye research and thus help discover new drugs and treatments for OHT/glaucoma. To that end, Section 8 (Chapters 43-48) provides some new information on modern techniques and technologies addressing some novel avenues of eye research delving into gene and cell

therapies and studying cell-to-cell communication via nanotubes and study of metabolomics of cells in the ANCe.

In conclusion, both clinical and basic science ocular researchers, graduate students, postdoctoral fellows, optometrists, and ophthalmologists should find this book to be a comprehensive and one-stop-resource for methodologies needed to study ocular structure and function of cells and tissues of the ANCe. The preservation of our most precious sense, eyesight, and its potential restoration in patients afflicted with blinding eye diseases such as OHT/glaucoma is a goal worthy of pursuit. We hope that this collection of protocols helps eye scientists achieve such audacious goals. We extend our thanks and gratitude to all the authors and the publisher for making this book a reality.

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OCULAR HYPERTENSION (OHT) AND GLAUCOMA AND REASONS FOR STUDYING THEM

CHAPTER 1

Basic Anatomy and Physiology of the Human Eye

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Abstract: The eye is an exquisitely designed sensory organ adapted to respond to ambient white light. It is composed of many specialized structures and cell types that have defined functions. Good vision is important for us to perceive our environment and to conduct our daily life activities. In simple terms, the eyes detect light and convert it into electrochemical impulses, which the brain decodes into the final images we perceive. Thus, in higher organisms, the eye is a complex optical system that collects light from the surrounding environment, regulates its intensity through the pupil, and focuses it *via* the lens to form an image at the back of the eye. This image is then converted into a set of electrical impulse signals transmitted *via* the retinal ganglion cells (neurons) and their axons (bundled into the optic nerve) to the thalamic and suprachiasmatic nuclei and, ultimately, to the visual cortex. Visual perception in these higher brain systems, therefore, is reliant on proper and optimal functioning of all components of the retina-optic nerve-brain structure system. Defects along this visual axis result in many types of eye diseases of which glaucoma (made up of many disorders) is one of the major leading causes of worldwide visual impairment that can lead to blindness. The eye structure and function at a high level are described in this chapter.

Keywords: Anatomy, Glaucoma, Light, Ocular diseases, Ocular hypertension, Physiology, Vision.

INTRODUCTION

As the key sensory organ involved in vision, the photoreceptors in the retina of the eye respond to the environmental light, and the optic nerve from retinal ganglion cells transmits this information to the brain's visual centers for processing. Due to its unique location and functions, the eye is isolated and some-

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what protected from the blood-vitreous, blood-retinal, and blood-aqueous compartments. Due to the latter features, the eye has distinctly unique pharmacodynamic and pharmacokinetic characteristics (Goodman and Gilman, 2023).

The eye globe comprises and is surrounded by three tissue layers named sclera, choroid, and retina, and the globe can be split into the anterior and the posterior segments. Within the anterior segment are the following tissues: cornea, lens, iris, trabecular meshwork, canal of Schlemm, and the ciliary body. Conversely, the posterior segment houses and/or is connected to the vitreous humor, retina, choroid, and optic nerve (Fig. **1**). Tissues that are involved in focusing the incoming light onto the retina include the cornea, iris, and lens. However, it is the multi-layered retina that converts the photons of light into chemical-electric signals, which are relayed to the brain thalamic and visual centers for image generation and visual perception. Meanwhile, physiological balance, appropriate intraocular pressure maintenance, and nourishment of ocular tissues are provided by the choroid, aqueous humor, vitreous humor, and the lacrimal gland system.

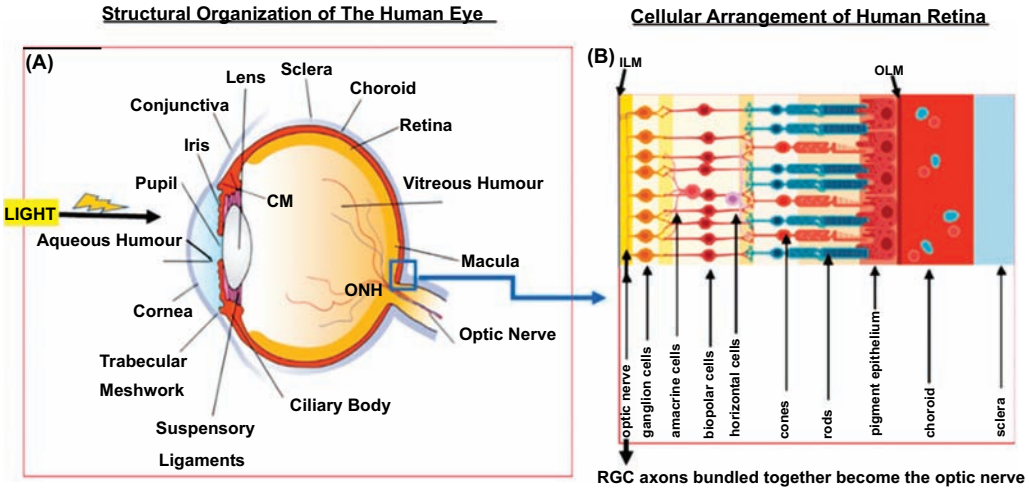

Fig. (1). Key structural elements of the human eye: anterior segment (**A**) and Posterior Segment (**P**).

EXTRAOCULAR STRUCTURES

Eye Lids

This specialized thin layer of skin is responsible for protecting the eye, with the inner portion lined with the palpebral portion of the conjunctiva, which keeps the eyes moist *via* the blink response. The eyelids also protect the eyeball from airborne pollutants, chemicals, and potential mechanical injuries. The cornea and

conjunctiva are highly innervated with sensory nerves, and they initiate the blink response. The oily film produced by the meibomian glands and the aqueous lacrimal secretion from the eyelids keep the cornea and conjunctiva moist and prevent excessive tear evaporation.

Lacrimal System

Secretory glands and excretory (nasolacrimal) ducts are part of the lacrimal system. This system includes lacrimal glands located in the temporal outer portion of the orbit innervated by the autonomic nerves and accessory glands located in the conjunctiva (Krause and Wolfring glands). The meibomian glands are located on the posterior and inferior surface to the eyelashes, whereas orifices of the glands are positioned posterior to the eyelashes and secrete oils that slow down tear evaporation. Capillary action and blinking permits the tears to flow through the lacrimal drainage system and out to the nasal cavities.

Orbit

The bony cavity of the skull that houses and protects the eyeball is the orbit. This structure has multiple fissures and foramina that house the nerves, muscles, and blood vessels. Connective tissue, adipose tissue, and six extraocular muscles align and support the eyes within the orbit to permit binocular vision. The retrobulbar region is immediately behind the eye.

OCULAR STRUCTURES (ANTERIOR SEGMENT)

Cornea

The cornea is composed of five layers (epithelium, bowman's membrane, stroma, Descemet's membrane, and endothelium) and is a transparent and avascular tissue. Six cell layers form the hydrophobic epithelial layer, which is fundamentally interconnected with the tear film. An average adult cornea is approximately 11.5 – 12.0 mm in diameter and 0.5 mm thick (Rüfer *et al*., 2005). The corneal limbus area receives the blood supply, and the tears and aqueous humor support the metabolic processes of the cornea. The cornea and the tear film are important in providing some of the important optical power of the eyes (Donaldson *et al*., 2017).

Limbus

The transitional zone between the cornea and the sclera, consisting of conjunctival epithelium, Schlemm's canal, and the trabecular meshwork, is the limbus. The conjunctival epithelium contains the corneal epithelium stem cells, tenons

CHAPTER 2

Introduction to Ocular Hypertension and Glaucoma and an Overview of Drug Screening Funnels for Discovering and Characterizing New Intraocular Pressure-Reducing Compounds

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Abstract: Many forms of glaucoma prevail in our aging population. However, all forms cause a characteristic optic nerve and retinal ganglion cell (RGC) degeneration, which results in visual impairment and can ultimately cause blindness. The most common types of glaucoma are open-angle glaucoma (OAG) and angle-closure glaucoma (ACG), which afflict over 75 million individuals worldwide. Both these types of glaucoma involve an abnormal accumulation of aqueous humor (AQH) in the anterior chamber of the eye. This raises the intraocular pressure (IOP) which radiates out to cause mechanical damage to tissues at the back of the eye. Whilst no cure is available, the elevated IOP (ocular hypertension; OHT) is treatable with topical ocularly instilled drugs that either slow down the production of AQH or open up the congested AQH outflow pathway, which involves digestion of accumulated extracellular matrix in and around the trabecular meshwork and/or the ciliary muscle bundles. Furthermore, surgery and/or implantation of AQH microshunts in the anterior chamber can alleviate the OHT. Due to its chronic nature, medicated eyedrops are usually the first-line treatments involving the use of prostaglandin/prostanoid FP- and/or EP2-receptor agonists. Combination products are used if one drug fails to lower and control the elevated IOP. Drug discovery of new receptor- or enzyme-targeted molecules for OHT/glaucoma treatment is still warranted since existing drugs have several deficiencies and produce ocular and/or systemic side effects. This chapter addresses various elements of ocular hypotensive drug discovery and development.

Keywords: Drug discovery, Glaucoma, Intraocular pressure, Ocular hypertension, Trabecular meshwork, Vision.

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INTRODUCTION

Eyesight is our most precious sense that is vital for our daily activities, learning, socializing, and ultimate survival. Unfortunately, millions to over a billion individuals worldwide suffer from various preventable visual impairments and eye diseases (WHO, 2019). One of the leading causes of vision loss is a constellation of neurodegenerative disorders of the eye called glaucoma, of which the four major classes are open-angle glaucoma (OAG), angle-closure glaucoma (ACG), secondary glaucomas (SGs) and normotensive glaucoma (NTG) (Tham *et al*., 2014; Weinreb *et al*., 2014; Sharif, 2017, 2018, 2021). Globally, there are currently more than 75 million patients who suffer from OAG and ACG (Tham *et al*., 2014). Whilst all types of glaucoma damage the optic nerve, retina, and visual centers in the brain, the underlying pathogenic culprit causing OAG, ACG, and SGs involves defects in the aqueous humor (AQH) drainage system (trabecular meshwork [TM] and Schlemm's canal [SC]) located in the anterior chamber of the eye (ANCe) (Grierson and Howes, 1987; Sacca *et al*., 2005; Izotti *et al*., 2011; Buffault *et al*., 2020; Fig. **1**). At a highly simplified level, the aging process and pathological accumulation of extracellular proteins and cellular debris (De Groef *et al*., 2016) within the TM and in the juxtacanalicular area slow down the egress of AQH, which raises the intraocular pressure (IOP). Since the AQH is constantly being produced by the ciliary epithelium (Civan and Macknight, 2004) and its drainage is reduced (Acott *et al*., 2020), the IOP continues to slowly increase. Over time, the elevated IOP's mechanical force is exerted on all tissues of the eye. The stretching and distorting pressure adversely affects the most vulnerable tissues at the back of the eye, where the retinal ganglion cell axons coalesce before exiting the eye to form the optic nerve. Over time, the effects of this abnormally raised pressure at the optic nerve head and lamina cribosa initiate local inflammation (Hernandez *et al*., 1989; Burgoyne *et al*., 2005; McElnea *et al*., 2011) (Fig. **2**). The secreted cytokines and proteases injure/kill the RGC axons and the cell bodies causing a loss of connectivity to the brain centers, which culminates in visual impairment (Gupta *et al*., 2007; Fig. **2**). In OAG and ACG, over decades of asymptomatic destruction of RGCs due to the imperceptibly increased IOP, the patient's eyesight continues to deteriorate (Gazzard *et al*., 2003). When over 600k RGCs are lost, the patient begins to notice the loss of visual contrast, reduced color vision, and patchy blind spots in the visual field (Crab *et al*., 2016). It is now critical to lower and control the IOP, the only modifiable and treatable biomarker for OAG, ACG, and SGs, in order to preserve the remaining eyesight (Heill *et al*., 2002; Kass *et al*., 2002). Many drugs, surgical techniques, and AQH drainage microshunts are available to reduce the IOP, but they all have various shortcomings (Newman-Casey *et al*., 2015; Sharif *et al*., 2023) that limit their clinical utility (Weinreb *et al*., 2014; Sharif, 2017, 2018, 2021; Sadruddin *et al*., 2019; Sharif *et al*., 2023). Therefore, there continues to be

an unmet medical need to find new treatment modalities for all types of glaucoma, not just IOP-lowering therapeutics but neuroprotective agents as well (Sharif, 2018, 2021).

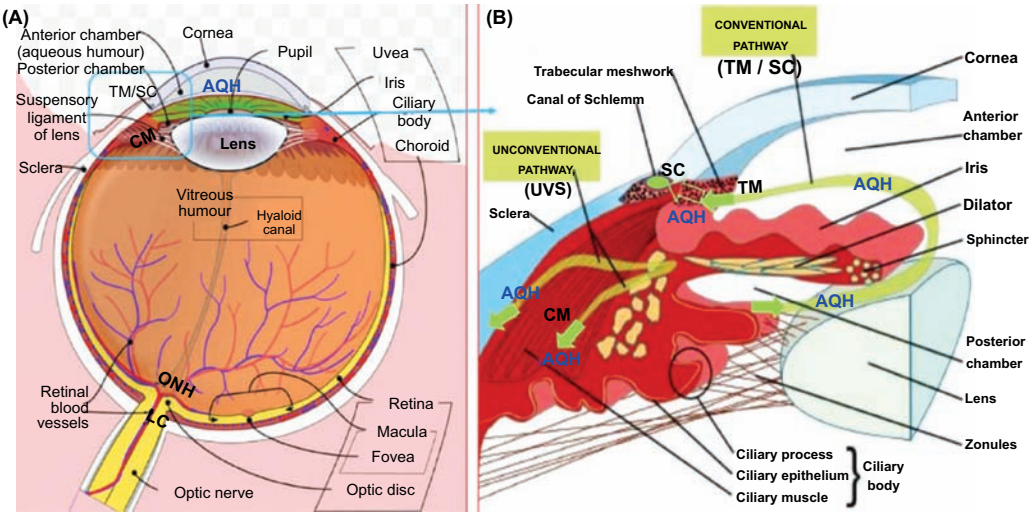

Fig. (1). The anatomy of the human eye (**A**) and the anterior chamber of the eye (**B**).

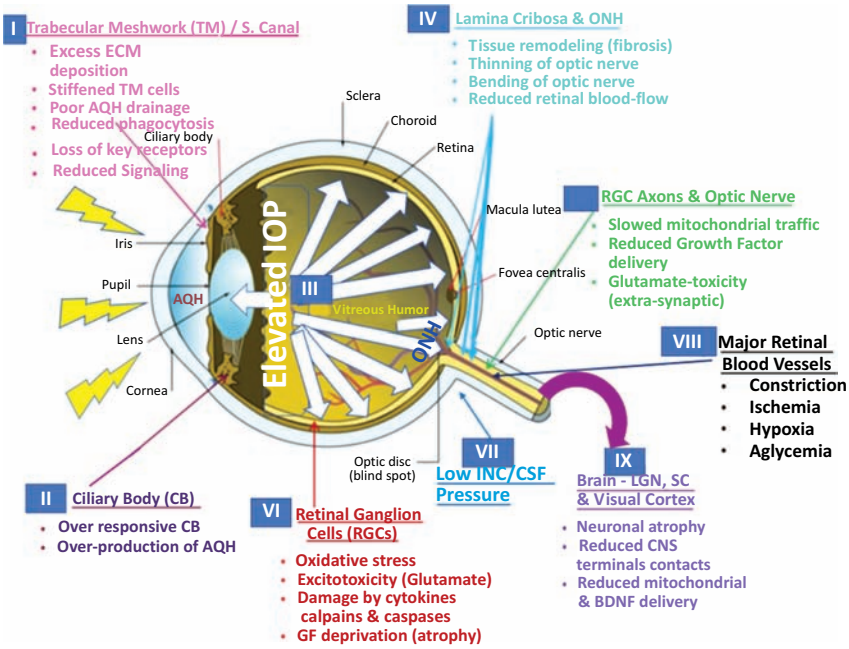

Fig. (2). The effects of chronically elevated IOP on ocular structures and functions that contribute to visual impairment.

CHAPTER 3

Protocols for Access to and Dose Selection of Selected IOP-Lowering Drugs

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Abstract: Described herein are high-level protocols to access selected small molecules with IOP-lowering properties when topically dosed as ophthalmic formulations, as well as dose selection for evaluation in appropriate animal models. Sources are provided for commercially available drugs, and high-level synthetic protocols are described for agents that are not commercially available. The agents described are approved for use in humans or have demonstrated robust pre-clinical activity. These are drawn from a diverse range of pharmacological classes, such as prostaglandin analogs, beta-adrenergic antagonists, carbonic anhydrase inhibitors, alpha-adrenergic agonists, and dual-activity prostaglandin-NO donors. Their physiological mechanisms of action have been characterized by the suppression of aqueous humor (AH) inflow, enhancement of AH conventional or uveoscleral outflow, and reduction of episcleral venous pressure.

Keywords: ATP-sensitive potassium channel openers, Adenosine receptor modulators, Alpha-adrenergic agonists, Antioxidant-NO donors, Beta-blockers, Carbonic anhydrase inhibitors, IOP lowering agents, Nitric oxide donors, Prostaglandins, Protein tyrosine phosphatase inhibitors, Rho kinase inhibitors.

INTRODUCTION

Given that the major glaucoma risk factor characterized to date is elevated intraocular pressure (IOP), frequently defined as sustained IOP values of ≥21mm Hg (Wijesinghe *et al*., 2022), agents that lower IOP have been the mainstay of agents used to treat glaucoma. The preferred delivery method is usually *via* topical application of an ophthalmic formulation of a small molecule, which has the advantage of achieving a high tissue concentration while limiting systemic ex-

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posure and subsequent side effects. Historic systemic administration of the carbonic anhydrase inhibitors acetazolamide and methazolamide are notable exceptions. Another is the investigative agent **FM-101**, an adenosine A3 receptor modulator which, unusually, is being studied as an oral formulation (see section 2 below).

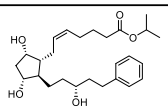
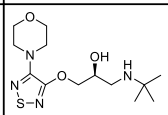
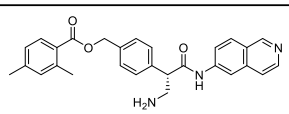
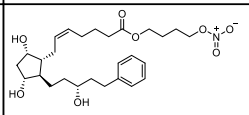
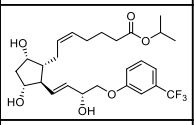
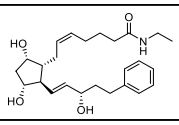
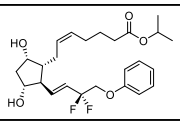
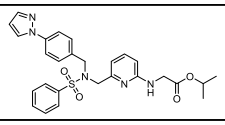
IOP-lowering drugs can typically be classified based on three physiological mechanisms of action: decreasing aqueous humor flow through the ciliary body, increasing aqueous outflow facility through the trabecular meshwork (conventional outflow), or increasing aqueous humor outflow through the uveoscleral pathway (non-conventional outflow). The additional mechanism of lowering episcleral venous pressure has been reported for the Rho kinase inhibitor netarsudil (Sit *et al*., 2021) and the ATP-sensitive potassium channel opener **QLS-101** (Chowdhury *et al*., 2022). Note that some agents lower IOP *via* more than one physiological mechanism of action.

The purpose of this chapter is to provide a high-level description of methods to access leading IOP-lowering drugs and drug candidates to further pre-clinical investigations. A more comprehensive analysis of topics such as sustained-release drug delivery systems, alternative therapies, discussion of the mechanism of action, and clinical effects of these drugs is reported by Liu *et al.* (Liu *et al*., 2022). In section 1, health authority-approved IOP-lowering agents that are commercially available, corresponding vendors, and the dosages reported to lower IOP in both rodent and large animal models are tabulated. Section 2 focuses on selected IOP-lowering agents that have not yet been approved by any health authority, with commercial access information provided or high-level synthetic protocols for those that are not commercially available.

Section 1: Access to Health Authority-Approved Iop-Lowering Agents

Table **1** is a list of drugs that health authorities have approved for lowering IOP in humans when they are applied as topical ophthalmic formulations. Prostaglandin FP receptor agonists are typically used as first-line agents (Klimko and Sharif, 2019), frequently with the addition of a beta-adrenergic antagonist (*e.g*., timolol), an alpha-adrenergic agonist (*e.g*., brimonidine), or a carbonic anhydrase inhibitor (*e.g*., brinzolamide) if IOP control is insufficient. A fixed combination of 2 different classes (*e.g*., fixed combination of brimonidine and brinzolamide; SIMBRINZA®) may be recommended as adjunctive therapy. The clinical impact of recently approved classes, such as dual-acting prostaglandin-nitric oxide donors and Rho kinase inhibitors, will be clarified over time.

Table 1A. Commercially available, regulatory-approved IOP-lowering compounds.

Name	Latanoprost (Xalatan)	Timolol	Netarsudil	Latanoprostene Bunod
Chemical structure				
CAS #	130209-82-4	26921-17-5 (maleate salt)	1422144-42-0 (dimesylate salt)	860005-21-6
Therapeutic class	Prostaglandin FP receptor agonist	β-adrenergic blocker	Rho kinase inhibitor	Prostaglandin FP receptor agonist and nitric oxide donor
Mechanism of action	Increase in uveoscleral AH outflow	Decrease in AH formation	Increase in AH conventional outflow and reduction in episcleral venous pressure	Increase in AH uveoscleral and conventional outflow
Doses	-			
Rodent models	Mouse-0.01% (Aihara *et al*., 2002) Rat- 50 ng (Husain *et al*., 2006) Rabbit-unresponsive	Mouse (Dexamethasone induced OHT model): - 3 µL of a 5-mg/mL solution (Whitlock *et al*., 2010); Rat (Hydrogel model)- 0.05% ;(Indian ink, laser coagulation) 0.5% (Yu *et al*., 2020); (Seki *et al*., 2005) Rabbit (Normotensive model): 0.5% (Lee *et al*., 2022)	Rabbit (Normotensive model): 0.04% (Lin *et al*., 2018)	Mouse (FP receptor knock-out mice: 0.006% (Saeki *et al*., 2009) Rabbit (Saline induced OHT): 0.036% (Krauss *et al*., 2011)
Large animal models	Dog-0.005% (Gelatt and MacKay, 2001) Monkey-0.005% (Serle *et al*., 1998)	Dog (Natural glaucoma)-0.5% (Plummer *et al*., 2006)	Dog (Normotensive model): 0.04% (Lin *et al*., 2018)	Dog (Natural glaucoma): 0.036% (Krauss *et al*., 2011) Monkey (Laser induced OHT): 0.036% (Krauss *et al*., 2011)
Commercial source	Cayman Chemical (www.caymanchem.com); ThermoFisher (www.thermofisher.com); Key Organics (www.keyorganics.net)	For maleate salt: Sigma-Aldrich (www.sigmaaldrich.com), Cayman Chemical (www.caymanchem.com)	For dimesylate salt: Sigma-Aldrich (www.sigmaaldrich.com), Abovchem (www.abovchem.com); Combi-Blocks (www.combi-blocks.com)	Sigma-Aldrich (www.sigmaaldrich.com), Cayman Chemical (www.caymanchem.com),
Chemical structure				
CAS #	157283-68-6	155206-00-1	209860-87-7	1187451-19-9
Therapeutic class	Prostaglandin FP receptor agonist	Prostaglandin FP receptor agonist	Prostaglandin FP receptor agonist	Prostaglandin EP_2 receptor agonist
Mechanism of action	Increase in AH uveoscleral outflow	Increase in AH uveoscleral outflow	Increase in AH uveoscleral outflow	Increase in AH conventional and uveoscleral outflow
Doses	-			

TARGET LOCALIZATION AND VERIFICATION STUDIES

CHAPTER 4

Protocols for Localizing Receptor mRNAs and Proteins by RT-PCR, *In Situ* Hybridization, and Autoradiography

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Abstract: Receptors, enzymes, transporters, and ion channels receive external signals and generate specific intracellular second messengers to mediate the biological response to the ligands that bind to the proteins on the cell membrane and/or to organelles inside the cells. In order to localize the potential target protein(s) that may be defective and/or those serving endogenous transmitter or drug activities in various tissues of the body, the use of reverse transcriptase-polymerase chain reaction and *in situ* hybridization coupled with quantitative receptor autoradiography are deployed. Such studies can be performed using isolated cells or postmortem tissues of animals and humans. Such techniques are powerful indicators of the presence or absence of the key receptors/enzymes and other protein targets that can help ensure target validation, localization, and engagement with exogenously delivered chemicals, drugs, antibodies, *etc.* These aspects are critical for drug discovery and can also indicate off-target side effects of drugs under study. These techniques will be described in this chapter.

Keywords: Transporters, Target engagement, Drug interaction, Enzymes, Ion channels, Receptors, Signal transduction, Target localization.

INTRODUCTION

The drug discovery and development process is a lengthy and expensive endeavor regardless of the disease being targeted. Therefore, it is important to know if and where a drug molecule binds with target and off-target tissues and cells of the body. Consequently, a number of techniques have been developed, validated, and utilized in order to study the interaction and functional engagement of endogenous

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chemicals (*e.g.*, neurotransmitters) and potential drug candidates (small compounds, peptides, antibodies, *etc.*) with their target proteins (receptor, enzyme, transporter, or ion channel protein). This chapter describes protocols to perform reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization (ISH) microscopy to obtain information about the presence/absence of the mRNAs of targeted proteins and their distribution. Autoradiography allows localization, visualization, and quantification of radioligand-protein complexes *in vitro* and *ex vivo*, depending on the study design and the expected outcome. These are important techniques to demonstrate the drug molecule-target protein interactions in cells and tissues of importance from an efficacy and potential safety perspective.

Prostaglandins (PGs) and serotonin (5-hydroxytryptamine; 5-HT) are endogenous transmitter substances that elicit their biological actions by interacting with specific cell membrane-bound receptors. Several sub-types of receptors for each class of transmitter have been identified and characterized using agonist and antagonist drugs. For instance, DP- (DP1-2), EP- (EP1-4), and FP-PG receptors exist, and receptor-selective drugs have been created that lower and control intraocular pressure (IOP) as treatments for many forms of glaucoma, a collection of neurodegenerative retinal/optic nerve/brain diseases (Weinreb *et al*., 2014; Sharif, 2018-2022). Thus, the free acid forms of FP-receptor agonists (*e.g.*, latanoprost and travoprost) engage with their cognate receptors to stimulate phosphoinositide turnover in the cytoplasm, which produces inositol phosphates and diacylglycerol (Sharif *et al*., 2023). These agents, in turn, liberate intracellular Ca^{2+} and lead to the phosphorylation of various enzymes and other proteins in the target cells. These activities culminate in the outflow of aqueous humor fluid (AQH) from both the unconventional uveoscleral outflow pathway (across the ciliary muscle and sclera) and, to some degree, from the conventional outflow pathway that involves trabecular meshwork/Schlemm's canal (TM/SC) and IOP reduction. Similarly, drugs that bind to EP2-receptors, for example, omidenepag (Sharif *et al*., 2023), raise intracellular cAMP levels that induce a plethora of additional intracellular reactions to enhance relaxation of TM and digest extracellular matrix to promote AQH outflow from the anterior chamber of the eye *via* the unconventional and conventional outflow pathways to reduce IOP (Sharif *et al*., 2023). A similar situation prevails for 5-HT2 receptors and subtypes. Hence, 5-HT2A-2C receptor-selective agonists exert strong ocular hypotensive actions when dosed topical ocularly in ocular hypertensive monkey eyes to suppress elevated IOP (Sharif et l., 2006; Sharif and Senchyna, 2006). Below are described some of the key methods to determine the presence/absence of the receptor mRNA and proteins for selected PGs and 5-HT, which serve as good examples for these ocular hypotensive activity mediators.

RT-PCR of Receptors in Eye Tissues of the Anterior Chamber

RT-PCR is a technique combining reverse transcription of RNA into DNA (in this context called complementary DNA or cDNA) and amplification of specific DNA targets using the polymerase chain reaction (PCR). It is primarily used to measure and quantify the amount of a specific RNA, which is accomplished by monitoring the amplifying reaction using fluorescence, a technique called real-time PCR, or, in other cases, quantitative PCR (qPCR). Combined RT-PCR and qPCR are utilized to assess gene expression and quantification of mRNAs. Some publications where receptor mRNAs were ascertained by RT-PCR include those by Ullmer *et al.* (1995), Senchyna *et al.* (2000), Kyveris *et al.* (2002), Sharif *et al.* (2002), Chidlow *et al.* (2004), and Sharif and Senchyna (2006). As examples, these publications provide the necessary procedures to determine mRNAs for FP- and TP- prostaglandin and serotonin receptors.

Isolation of Total RNA

- Aseptic isolation of total RNA from tissues isolated from the human cadaveric anterior chamber should be performed with the RNeasy Midi Kit (QIAGEN Inc., Mississauga, Ontario, Canada).
- Determination of the concentration and purity of total RNA should be performed by UV light absorption on a GeneQuant pro RNA/DNA calculator (Biochrom Ltd.; Cambridge, UK). If optical densities at 260 nm/280 nm are lower than 1.6, the preparations should be discarded.
- Use electrophoresis to determine the presence of intact RNA by loading it on 1% agarose-formaldehyde gels. RNA isolates can be accepted to be intact if the UV fluorescence of the 28S rRNA band is twice the intensity of the 18S rRNA band and if no UV fluorescence is found below the 18 rRNA band.

mRNA Detection by RT-PCR

- Synthesis of sense and antisense-specific primers ought to be based on an intron-exon boundary design in order to distinguish between the amplification of mRNA and genomic DNA and should be based on reported/published cDNA sequences. BLAST retrieval of human cDNA sequences (based on GenBank searches) should be used to design other primers.
- Procedures used to determine the RT-PCR-based detection of mRNAs for various 5-HT receptors in human ocular tissues will be illustrated (Fig. **1**).
- Total RNA (25 to 97 µg) should be isolated from the ocular tissue samples followed by conversion to cDNA in a 10 µl reverse transcription reaction solution containing 0.5 mg of total RNA of each sample, 1X first strand buffer (75 mM KCl; 50 mM Tris-HCl, pH 8.3; 3.0 mM $MgCl_2$), 1.7 mM $MgCl_2$, 1 mM each dNTP, 10 mM dTT, 2.5 mM oligo (dT)18, and 5 U/µl of SuperScript™ II

CHAPTER 5

Protocol for Generating and Utilizing Ocular Tissue Homogenates for Receptor Binding Studies

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Abstract: Ocular tissues play a vital role in various physiological processes for visual processing and are critical for understanding ocular diseases and drug development. By homogenizing ocular tissues, researchers can obtain a representative sample of ocular components, allowing for the investigation of receptor binding characteristics. This method provides valuable insights into the molecular interactions between ligands and ocular receptors, aiding in the development of novel therapeutic interventions. The utilization of ocular tissue homogenates offers a practical approach to studying ocular receptor binding and holds significant potential for advancing our understanding of ocular pharmacology and the development of targeted ocular therapies. The role of prostaglandins (PGs) in regulating IOP was studied several years prior to identifying prostaglandin receptors in the eye. Quantitative autoradiography, *in situ* hybridization, immunohistochemistry, and RT-PCR confirmed EP and FP receptors presence in the ciliary epithelium, cornea, conjunctiva, iris sphincter muscle, longitudinal ciliary muscle, retinal ganglion cells, trabecular meshwork, sclera, Muller cells, and optic nerves. For illustrative purposes, the aim of this chapter is to describe the key elements of experimental protocols used to study the pharmacological characteristics and regional distribution of prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) receptor binding sites in membranes isolated from bovine iris ciliary body.

Keywords: Bovine, Iris ciliary body, Ocular receptor binding sites, Prostaglandins, Sphincter.

INTRODUCTION

As our population ages, the incidence of devastating eye diseases increases. It has been estimated that more than 65 million people suffer from glaucoma worldwide (Quigley and Broman. Br J Ophthalmol 90:262–267, 2006). In addition, developing treatment programs for these various diseases that could affect our eyesight is also critical. One of the most effective treatments we have is in the development of specific drugs that can be used to target various components of

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the mechanisms that lead to ocular disease. Understanding basic principles of the pharmacology of the eye is important if one seeks to develop effective drug treatments. The eye is a complex sense organ that is composed of many unique types of tissues involved in light sensing and the visual transduction cascade. Within these tissues are cells that contain various classes of proteins that can act as receptors for pharmacological drugs to interact with and produce a measurable cellular/signaling response that leads to the final biological response (*e.g.*, tissue contraction/relaxation, transmitter release, and intraocular pressure reduction [IOP]).

The role of prostaglandins (PGs) in regulating IOP was studied several years prior to identifying prostaglandin receptors in the eye. Early studies revealed that topical ocular dosing of many different classes of prostaglandins produces a diverse set of responses, including a biphasic response in terms of IOP: an initial short-term increase in IOP followed by a sustained decrease in IOP (Camras *et al.* 1977; Starr 1971). These were dose-, time- and species-dependent. Prostaglandins are ubiquitous, and the types produced in a given cell are dependent on the expression profile of the prostaglandin synthetic enzymes in that particular cell. Prostaglandins are autocrine or juxtacrine modulators that have diverse pharmacological effects on the central nervous, the cardiovascular, gastrointestinal, and visual systems.

Quantitative autoradiography, *in situ* hybridization, immunohistochemistry, and RT-PCR confirmed EP and FP receptors presence and a differential distribution in the ciliary epithelium, cornea, conjunctiva, iris sphincter muscle, longitudinal ciliary muscle, retinal ganglion cells, trabecular meshwork, sclera, Muller cells, and optic nerves (Anthony *et al.* 2001; Davis and Sharif 1999; Matsuo and Cynader 1992; Ocklind *et al.* 1996; Schlotzer-Schrehardt *et al.* 2002). In the eye, topical ocular prostaglandins (PGs), in particular PGE2 and PGF2 alpha, may induce vasodilation, disruption of the blood-aqueous barrier, and IOP reduction, depending on the species.

The purpose of this chapter is to describe the key elements of experimental protocols to study the characteristics and regional distribution of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) in membrane from isolated bovine iris ciliary bodies. Currently, 5 types of prostanoid receptors have been classified based on their affinity for naturally occurring prostanoids (Kennedy I *et al.* 1982; Coleman *et al.* 1984), *i.e.*, PGE_2, $PGF_{2\alpha}$, PGD_2, thromboxane, and prostacyclin. Understanding the specific prostanoid receptor distribution will provide a better understanding of the ocular effects of PGs and their analogs in terms of their on-target and off-target effects. Below are described methods to study the receptor binding characteristics of [^{3}H]-PGE_2 and [^{3}H]-PGF_{2a} to cell membrane

homogenates of the bovine iris-ciliary body as originally described by Csukas *et al.* (1993). The same types of procedures can be utilized to dissect human eyeballs and conduct receptor binding experiments to discern the pharmacology of other receptor types in ocular tissues of other species using different radioligands (Chidlow *et al*., 1995).

Experimental Protocols

Tissue Membrane Preparation

- Obtain freshly isolated bovine eyes transported on ice from the abattoir.
- Dissect the iris ciliary body circumferentially into three parts: sphincter muscle, ciliary body and processes, and the rest of the iris. This is achieved by dissecting out the sphincter and then the ciliary body with attached processes. The rest of the iris will include the blood vessels and the dilator muscle, with some sphincter muscle and ciliary body fragments (Csukas *et al.* 1993).
- Maintain the tissues at 2-4°C and homogenize each tissue separately in 50mM tris buffer at a pH of 7.5, which should contain cyclooxygenase (1μM flurbiprofen), protease (0.43mM PMSF), and 0.01% soybean trypsin inhibitors to remove endogenous PGs. Add 1mM EGTA to the homogenization buffer (Csukas *et al.* 1993).
- Obtain tissue membranes after three-stage high-speed centrifugation (Bhattacherjee *et al.* 1990). Centrifuge the tubes in an ultracentrifuge for 60 min at 120,000 g, discard the supernatant (Chidlow *et al*.,1995), then centrifuge the homogenate at 300g for 10 minutes at 4°C, and aspirate away the supernatant.
- Coarsely mince the pellets and place them in a glass scintillation vial containing 2M sucrose and glass beads and disperse the pellet further to homogeneity as much as possible. This procedure is gentler than using an electrical homogenizer.
- Now add this suspension to fresh ultracentrifuge tubes and gently overlay with fresh buffer solution.
- Centrifuge tubes for 90 min at 120,000 g and isolate the turbid layer formed at the sucrose-buffer interface, which contains the cell membranes, using a Pasteur pipette.
- Transfer the isolated fraction into fresh ultracentrifuge tubes and centrifuge for 1hr at 120,000 g to compact the membranes.
- Now, resuspend the resulting pellet in fresh receptor binding buffer to the appropriate protein concentration or membrane concentration (*e.g*., 150-400 Mm/ml).

CHAPTER 6

Protocols for Visualizing and Quantifying Receptors in Ocular Tissues Involved in Aqueous Humor Inflow and Outflow

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Abstract: Glaucomatous optic neuropathy (GON) globally afflicts millions of patients and gradually but progressively robs them of their eyesight over decades of undetected and undiagnosed disease progression. Chronically raised intraocular pressure (IOP), also known as ocular hypertension (OHT), is most significantly associated with the development of GON and is the only modifiable and treatable biomarker for the most prevalent form of glaucoma, open-angle glaucoma (OAG). There continues to be a need to discover and introduce new drugs with good therapeutic indices in the clinical management of OAG. Potential targets are being discovered from genetic studies, but their localization and verification in the target tissues and cells in the anterior chamber (ANC) of the eye need to be determined before embarking on initiating drug discovery campaigns. Several techniques have been developed for target verification. This chapter deals with quantitative autoradiographic procedures to visualize receptor targets for drug candidates and existing marketed medicines treating OHT and OAG.

Keywords: Autoradiography, Drug targets, Enzymes, Immunohistochemistry, Receptors.

INTRODUCTION

Several million people worldwide suffer from neurodegenerative ocular diseases grouped together as glaucoma. The most prevalent form is open-angle glaucoma (OAG), in which ocular hypertension (OHT) caused by excess accumulation of aqueous humor (AQH) in the anterior chamber (ANC) of the eye is the major culprit and risk factor (Weinreb *et al*., 2014; Sharif, 2021-2023). The elevated intraocular pressure (IOP) damages the delicate retinal structures and the optic

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nerve at the rear of the globe and kills the highly sensitive and critically important retinal ganglion cells (RGCs). These damaging events occur over decades, but once half of the original RGCs have been lost, the connections between the retina and the brain structures involved in visual perception and eyesight are also halved, and the patient starts to notice significant visual impairment (Sharif, 2021-2023). In order to stave off further damage, the elevated IOP has to be reduced using pharmaceuticals, drainage-device implants, or via surgery. The most cost-effective means is topical ocular administration of IOP-lowering medications (Bucolo *et al*., 2018; Sharif, 2021-2023).

Whilst a number of drugs are available for lowering and controlling IOP (Bucolo *et al*., 2018; Sharif, 2021; Sharif *et al*., 2023), they impart significant side effects and their pharmacological and pharmacokinetic properties are somewhat undesirable and should be improved. New drug discovery is an active and important discipline in this endeavor. However, whilst new therapeutic targets are being discovered via genome-wide associated studies, the localization and verification of potential receptors, enzymes, ion channels, and transporters in the ANC of the eye, which may mediate beneficial effects through such proteins, are often lacking. Even though immunohistochemistry (Sharif *et al*., 2014) is a useful technique to define the distribution of receptors, autoradiography is perhaps easier to perform and is quantitative when coupled with computerized image analysis (Sharif and Hughes, 1989; Sharif and Eglen, 1993; Davis and Sharif, 1999; Sharif *et al*., 1995, 1988, 1999, 2000, 2001, 2004, 2014; Sharif and Lewis, 1989; Sharif, 1986, 1989, 1996).

Protocols for Performing Prostaglandin Receptor Autoradiography *In Vitro*

Many neurotransmitter and drug receptors have been visualized on thin sections of animal and human postmortem eyes over the years from a basic research perspective. Most studies have simply reported the presence or absence of these target receptor or enzyme proteins (Pilotte *et al*., 1984). Over the intervening years, the use of powerful image analysis technologies and techniques has successfully permitted quantitative digital subtraction of autoradiographic images to provide high specific receptor binding profiles of radiolabeled molecules in ocular tissues and tissues from other body organs. Additional progress has been made in performing kinetic and drug-concentration-based titration to help determine receptor binding affinities in tissues that are too small to isolate for traditional receptor binding techniques described elsewhere in this book and in many publications (Sharif and Hughes, 1989; Davis and Sharif, 1999; Sharif *et al*., 2000; Green and Sambrook, 2020; Hansen *et al*., 2022; Kecheliev *et al*., 2022; Mikkelsen *et al*., 2022; Mitsukawa *et al*., 2022; Murgas *et al*., 2022; Neuman *et al*., 2022; Voss *et al*., 2022; Waldman *et al*., 2022; Ganaglio *et al*., 2023;

Krogsback *et al*., 2023). An example of localizing and quantitative autoradiography is presented below with emphasis on FP-and DP-prostaglandin receptors in human eye sections *in vitro* (Fig. **1**).

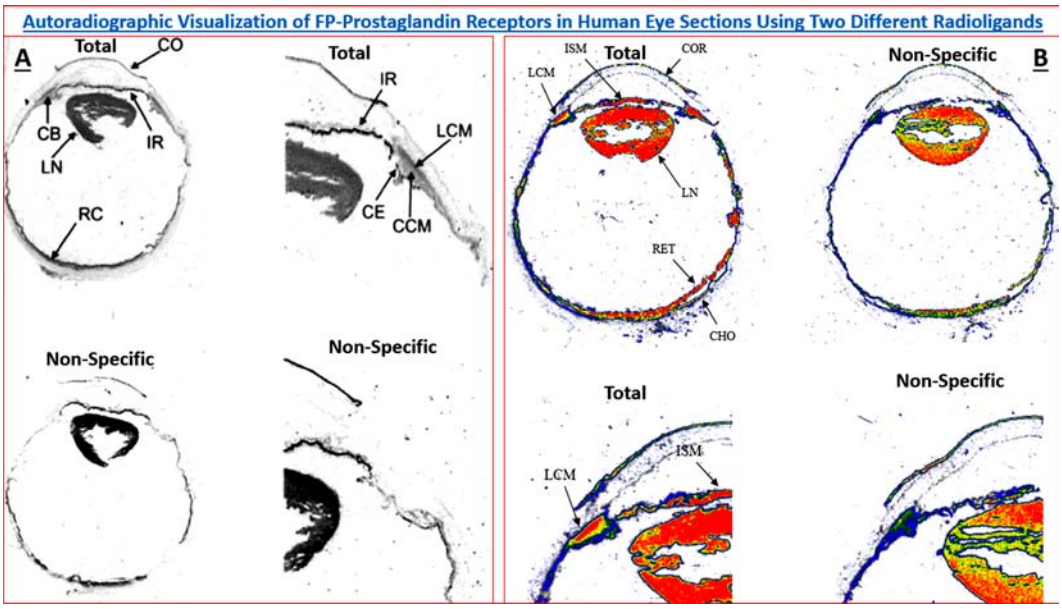

Fig. (1). Visualization of FP-prostaglandin receptors in postmortem human eye sections by autoradiographic means using [^{3}H]-travoprost acid as the radioligand, shown in black and white as images appeared on the tritium-sensitive Hyperfilm (A). In the next panel (B) is the [^{3}H]$PGF_{2\alpha}$ binding to human postmortem eye tissues, where the autoradiographic images have been pseudo-color coded by the Optiquant® software on an Agfa Arcus® scanner image analysis system to show the relative density of the receptors present using the visual spectrum of colors (blue to red: with increasing density). Note the high density of specific binding to the longitudinal ciliary muscle (LCM), iris sphincter muscle (ISM), and retina (RET). The retina is distinguished from the choroid (CHO) in this section as it is pulled away from the choroid during sectioning. Also, note low levels of specific binding to lens (LN). Enlarged views in C (total binding) and D (nonspecific) show weak specific binding to the ciliary epithelium and ciliary process (CP). The cornea (COR) is barely visible in C. Magnifications pertaining to panels A and B, which were 3.5x, while panels C and D were 9.1x of the original images.

- Obtain enucleated postmortem human eye globes within 4-12 hours post-death from local eye banks and keep them on ice.
- Embed a globe in Tissue-Tek O.C.T embedding material and leave it in the cryostat for a few hours to fully freeze.
- Cut tissue sections (20 um) at 17C on a freezing microtome and collect them on gelatinized glass microscope slides. Store slides in a desiccator at -20°C for up to 1 week or when needed for experiments. It is recommended that these sections be used within 1-2 months after cutting.
- For the receptor binding experiments, thaw the slides/sections at room temp (22°C) and then preincubate them by dipping whole racks of slides in sufficient

CHAPTER 7

Protocols for Prostaglandin Receptor Localization in Eye Tissues Using Immunohistochemistry

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Abstract: This chapter describes the detailed protocols for light and electron microscopic immunolocalization of prostaglandin (PG) receptors in ocular cells and tissues using immunofluorescence labeling of frozen sections, immunoperoxidase labeling of paraffin sections, and immunogold labeling of ultrathin sections. Unlike Western blotting analysis, immunohistochemistry allows for fine mapping of protein localization in intact tissues and cells, including assessment of relative abundance using image analysis software programs. Precise receptor subtype distribution is important for understanding mechanisms of action of therapeutic PG analogs, for identifying targets for novel treatments using subtype-specific high-affinity receptor agonists, and for improving safety profiles and minimizing potential side effects of anti-glaucoma pharmacotherapies.

Keywords: Trabecular meshwork, Anterior segment, EP receptor, FP receptor, Glaucoma, Human eye, Immunohistochemistry, Prostaglandin receptors.

INTRODUCTION

Prostaglandins (PGs) are arachidonic acid-derived lipid mediators that regulate a great variety of cellular functions by acting on specific G-protein-linked cell surface receptors expressed in the majority of mammalian cells. The prostanoid receptor subfamily comprises nine known members, including DP1-2 (PGD_2 receptors), EP1–4 (PGE_2 receptors), FP ($PGF_{2\alpha}$ receptor), IP (prostacyclin I2 receptor), and TP (thromboxane A_2 receptor), which are classified according to the prostanoid ligand that binds with greatest affinity (Coleman *et al.* 1994). Activation of these receptors by their respective ligands results in an elevation (DP-, EP2-, EP4-, and IP-receptors) or reduction (EP3-receptors) of cAMP or in the production of intracellular inositol phosphates that, in turn, raise intracellular

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Ca^{2+} concentrations (EP1, FP- and TP-receptors) (Sharif *et al.* 2023). These changes in cAMP and Ca^{2+} trigger downstream signal transduction pathways, such as phosphorylation and activation of various kinases, transducing prostanoid receptor activation into biological effects. The precise roles of prostanoid receptors in physiologic and pathologic conditions depend on multiple factors, including cellular context, receptor expression profile, ligand affinity, and coupling to various signal transduction pathways. This complexity is highlighted by the diverse and often opposing effects of PGs, which, for example, commonly act as pro-inflammatory mediators but can also have anti-inflammatory properties in certain settings (Hata and Breyer 2004).

In the eye, PG receptors have been implicated in the regulation of inflammatory processes, immune responses, blood flow, and intraocular pressure (IOP) (Doucette and Walter 2017). FP, DP, and EP receptor subtypes have been identified in virtually all human ocular tissues, including cornea, sclera, conjunctiva, trabecular meshwork, Schlemm's canal, ciliary body and ciliary muscle, iris, lens, choroid and retina (Woodward *et al.* 1997; Mukhopadhyay *et al.* 1997, 2001; Kamphuis *et al.* 2004; Schlötzer-Schrehardt *et al.* 2002; Biswas *et al.* 2004; Doucette and Walter 2017) using molecular biologic and immunohistochemical methods (Fig. **1**). FP receptors were found abundantly in the ciliary muscle, particularly the circular portion, and ciliary epithelium, as well as in dilator and sphincter muscles of the iris. EP1-4 receptor transcripts and proteins were found to be widely expressed in many ocular cell types, predominantly corneal epithelium, endothelium and keratocytes, trabecular endothelium, iris epithelium, vascular endothelium and stromal cells, and ciliary epithelium. DP receptors were also localized at high concentrations to the epithelium of the ciliary processes, ciliary muscle, iris tissue, choroid, and retina using quantitative autoradiography (Sharif *et al.* 2005). The TP receptor was also found to be expressed in the trabecular meshwork (Kamphuis *et al.* 2001). Prostaglandin receptor expression in ocular tissues may be altered by certain stimuli, including elevated IOP (Schneemann *et al.* 2002) and in certain pathological conditions (Singh *et al.* 2023; Ueta *et al.* 2011).

Topically administered PGs and their analogs have been shown to significantly lower IOP and have thus evolved as first-line drugs in glaucoma therapy. Classical PG analogs like latanoprost lower IOP *via* the FP receptor, principally by increasing aqueous outflow facility through the uveoscleral pathway by the activation of matrix metalloproteinases and extracellular matrix turnover in the ciliary muscle and sclera (Sharif *et al.* 2023). However, FP receptor agonists may also cause ocular side effects, such as conjunctival hyperemia as well as iris and periorbital skin darkening. Moreover, the effect of FP receptor agonists on the conventional outflow pathway and on trabecular endothelial cells has been less

clear. In fact, FP and EP2 receptor agonists have been shown to exert opposing effects on trabecular cell contraction, myofibroblast transformation, and collagen deposition (Kalouche *et al.* 2016). More recently, a novel dual FP/EP3 receptor agonist, sepetaprost (ONO-9054 or DE-126), and a selective non-PG prostanoid EP2 receptor agonist, omidenepag isopropyl (OMDI), have also demonstrated robust IOP-reducing activities by enhancing both the uveoscleral and the conventional trabecular outflow pathways (Yamagishi-Kimura *et al.* 2022; Sharif *et al.* 2023).

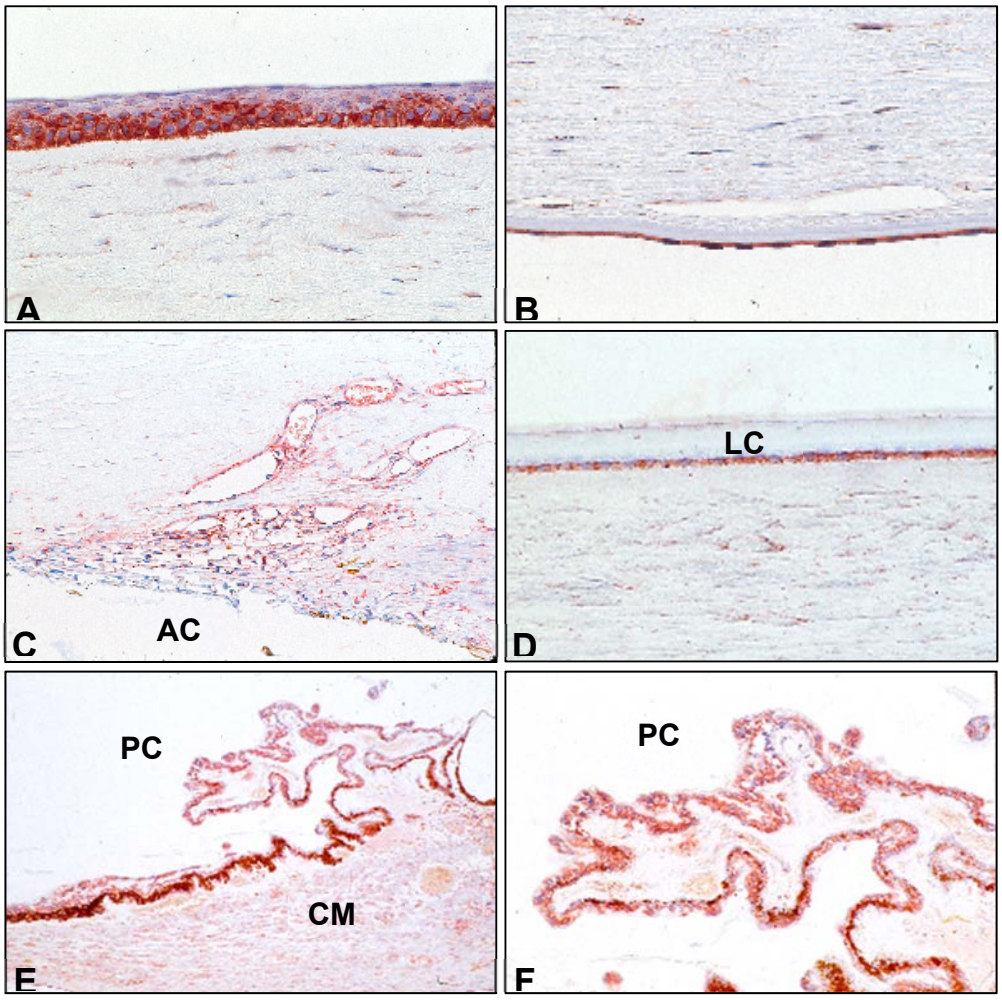

Fig. (1). Immunolocalization of the EP1 receptor in paraffin-embedded sections of human anterior segment tissues. Positive immunoreactions can be seen in the corneal epithelium (**A**), the corneal endothelium (**B**), the trabecular meshwork, Schlemm's canal, collector channels and intrascleral aqueous veins (**C**), the lens epithelium (**D**), the ciliary muscle and the ciliary epithelium (**E,F**); (AC, anterior chamber; CM, ciliary muscle; LC, lens capsule; PC, posterior chamber).

OCULAR CELL TYPES AND SOURCES FOR ISOLATION AND CHARACTERIZATION

CHAPTER 8

Isolation and Use of Trabecular Meshwork Cells

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Abstract: As described elsewhere in the book, ocular hypertension (OHT) is a major risk factor for the development and progression of open-angle, closed-angle, and secondary forms of glaucoma. OHT or elevated intraocular pressure (IOP) is caused when the major aqueous humor (AQH)-drainage pathway, the trabecular meshwork (TM), and Schlemm's canal (SC) are slowly occluded by progressive deposition of extracellular matrix in and around the latter structures. In order to study the structure and function of the TM, most researchers isolate this tissue from different species, including from post-mortem human eyes, and either use the whole tissue (*e.g*., in contraction/relaxation assays) or isolate individual single cells and propagate them under tissue culture conditions. These cells can then be used for studying the morphological, biochemical, and pharmacological changes induced by various insults to simulate OHT/glaucomatous disease states or in response to compounds of interest utilizing various functional readouts.

Keywords: Functional assays, Intrinsic activity, Intraocular pressure, Ocular hypertension, Potency, Prostaglandin, Trabecular meshwork.

INTRODUCTION

Trabecular meshwork (TM), in conjunction with Schlemm's canal (SC), represents the major outflow drainage pathway for aqueous humor (AQH) in the anterior chamber of the eye (ANCe). Under normal circumstances, there is an equal volume of AQH produced by the ciliary body and exiting the ANCe to maintain a steady intraocular pressure (IOP) (Acott *et al*., 2020). This ensures that the AQH provides the tissue lining the ANCe nourishment, oxygen, and anti-microbial substances to ensure homeostasis and good health of all those tissues and their cells. The constant IOP at normal levels (14-21 mmHg) also ensures a steady shape of the eyeball. However, in advancing age and under pathological

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conditions, as in different forms of glaucoma, the TM and the juxtacanalicular area experience an abnormal deposition of the extracellular matrix, which clogs these tissues and slow down the AQH egress from the ANCe (Rhee *et al*., 2003; Weinreb *et al*., 2014). As the AQH production continues, accumulating AQH fluid in the ANCe raises the IOP. Whilst this happens slowly and can take decades to manifest, even small increases in IOP can begin to damage the retinal ganglion cells and their axons (which form the optic nerve), leading to vision loss. There is a direct correlation between elevation of IOP and RGC loss and visual impairment (Heill *et al*., 2002; Kass *et al*., 2002). Therefore, it is important to diagnose and treat the OHT with pharmaceutical, device, or surgical interventions (Weinreb *et al*., 2014; Sharif, 2018, 2021) to lower and control the IOP.

Since the TM is the key tissue providing resistance to AQH drainage (Acott *et al*., 2020), it has been and continues to be the major tissue of interest linking OHT/glaucoma to visual impairment and for studying effects of glaucomatous insults on the cell morphology, biochemistry, and pharmacology. These cells have also been successfully used to profile compounds of interest that may possess IOP-lowering activities (Sharif *et al*., 2018, 2021). Primary TM cells have been isolated from the eyes of many mammalian species, cultured, and propagated over at least a few passages for the latter purposes (Keller *et al*., 2013, 2018). In some cases, the cells have been immortalized, and these have proven useful for receptor characterization in a functional assay system (Pang *et al*., 1994). This chapter aims to provide guidance on the isolation and culturing of primary TM cells, as recently detailed in a consensus publication (Keller *et al*., 2018). Readers are highly encouraged to consult the latter paper and the bibliography therein for an excellent background and other important aspects of the current topic.

Procedures for TM Isolation

Most researchers isolate TM cells from whole globes or anterior segments of the eye of a variety of animals and post-mortem human subjects. Corneal transplant corneal rim discards can also be utilized. Storage of tissues is preferably performed on ice in a humidified container with phosphate-buffered saline (PBS) or in Optisol, a specialized fluid that maintains corneal health. Post-mortem human eyeballs can be obtained from local eye banks under strict quality and safety conditions and should be carefully handled and placed in ice or brought to the lab in corneal transplant-solution. They must be serologically tested and worked on in a biological safety laminar flow hood. Isolated eyeballs of large animals such as cattle and pigs can be obtained fresh from local abattoirs and brought back to the lab in/on ice. Non-human primate tissue is often only obtained from the institutional animal husbandry department (vivarium) under strict IUCUC regulations. All eyeballs and tissues derived from the latter species should

be handled carefully and under stringent safety procedures as they may contain a variety of infectious viruses, bacteria, and perhaps even fungi. As such, researchers are encouraged to sterilize the eyeballs using ethanol (at least 70%) and/or an iodine-based antiseptic fluid to get rid of any infective agents. However, before dissection begins, ensure that the sterilizing fluids are removed by a series of rinses with sterile PBS. Since newly isolated cell viability and yield are time-dependent, the time elapsed from eyeball harvesting to tissue and cell isolation should be minimized as much as possible.

- Equatorially bisect the whole eyeballs using fresh, clean, and sharp scissors, a scalpel, or a single-edged razor blade.
- Under a well-illuminated binocular dissecting microscope, carefully dissect away ciliary processes, iris, and lens. In this case, cutting the anterior segment into many radial wedges in order to observe a flatter tissue surface and see the positioning of the TM/SC is deemed useful.
- Under the dissecting microscope, tip upwards the cut surface to see the SC and the TM. Now, isolate the TM from the tissue wedges using the techniques described in detail by Keller *et al.* (2018). The pooled TM tissue from several donor eyes can then be used to isolate the TM cells for culturing purposes.

TM Cell Isolation Procedures

- Place the dissected TM tissue strips (*e.g.*, each 2–5 mm in length, cumulatively about 25–35 mm as per the circumference of the human TM) into one well of a six-well culture plate and then carefully add the culture medium. Smaller 16 mm well plates can be used if necessary, but in this case, only 4–6 TM strips are placed in each well.
- Maintain the TM cultures undisturbed at 37°C in the humidified and 5% CO_2-controlled incubator for 1–2 weeks so that TM cells can migrate out of the TM explants and attach to the bottom of the well.
- As the TM cells migrate out, the TM strips should be moved into new plates to harvest more TM cells. The culture medium should be carefully changed every 3–5 days without disturbing the tissue strips. The TM cells obtained from a single individual (animal or human donor eye) should be described as cell strains.
- Unfortunately, a common issue encountered during the harvest of TM cells from the incubated TM explants is that the latter float up from the bottom of the plate well, especially when the culture medium is changed, and the cells can die. A remedy utilized to obviate this problem is to coat the culture plates with collagen or gelatin such that the cells and tissue strips can attach to the well-bottom surface and remain attached. Another solution for this problem is to place a sterile coverslip on top of the TM implant such that the tissue remains in contact

CHAPTER 9

Protocols for TM Cell Replacement Using Differentiated TM-Like Induced Pluripotent Stem Cells (iPSC)

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Abstract: Induced pluripotent stem cells are differentiated into TM-like iPSCs and transplanted into a cell depletion glaucomatous cell loss model. This restores the capability of this cell loss-perfused human anterior segment model to respond to a 2X pressure challenge with a normal IOP homeostatic response. Glaucomatous anterior segments cannot adequately respond to a 2x pressure challenge. Herein, we present protocols for working with human iPSCs, differentiating iPSCs into TM-like iPSCs, establishing the saponin TM cell depletion perfusion model for cell loss in the anterior segment, testing IOP homeostatic response, transplanting TM-like iPSCs into perfused human anterior segments, and evaluating transplanted cell integration. These protocols should facilitate similar studies on glaucomatous anterior segments and translation of these approaches to treat glaucomatous TM cell loss and their compromised IOP homeostatic response.

Keywords: Glaucoma cell loss, Intraocular pressure homeostasis, Induced pluripotent stem cells (iPSCs), Stem cell differentiation, TM cell replacement.

INTRODUCTION

Some years ago, a putative trabecular meshwork (TM) stem cell population was identified anterior to the filtering TM beneath Schwalbe's line inserted into the peripheral cornea, a region called the "TM insert". (Acott, *et al*., 1989) Resting human anterior segments exhibited low levels of cell division in this insert region, and treatment with laser trabeculoplasty approximately doubled this division rate within the first 24 hours. Pulse-chase studies showed that approximately 50% of the freshly divided cells then migrated out and repopulated the laser burn sites (Acott *et al*., 1989).

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Alvarado *et al*. (1981, 1984) and Grierson and Howe (1987) assessed TM cell density in electron micrographs and found a significant loss of cellularity with age and a much more pronounced TM cell loss in age-matched glaucomatous TMs. Abu-Hassan *et al*. (2015) developed a saponin detergent-based TM cell depletion model for glaucomatous loss in the anterior segment and found that a 30% TM cell depletion knocked out the intraocular pressure (IOP) homeostatic response to a 2x pressure challenge (Acott *et al*., 2014, 2016, 2021). Raghunathan *et al*. (2018) showed that glaucomatous anterior segments are also unable to mount an IOP homeostatic response to a 2x pressure challenge. When Abu-Hassan *et al*. (2015) added TM cells back to the saponin cell depletion model, the IOP homeostatic response was restored.

Based on the putative insert stem cell population and the glaucomatous cell loss with associated loss of IOP homeostatic responsiveness, Kelley *et al*. (2009) proposed that stem cells could be added to glaucomatous eyes and might restore IOP homeostatic capability. Following up on this hypothesis, a method for differentiating human iPSCs from TM-like iPSCs and then transplanting them into the saponin TM cell depletion human anterior segment model was established (Abu-Hassan, 2015). After transplantation and attachment of these TM-like iPSCs into the saponin TM cell depletion model, the IOP homeostatic response to a 2x pressure challenge was restored (Abu-Hassan, 2015). Variations of these studies by others have been conducted by using different models and efficacy assessments (Du *et al*., 2012, 2013; Zhu *et al*., 2016, 2017).

Experimental Protocols

Preparation of iPSC Embryoid Bodies (EBs)

a. Human iPS cells may be obtained from WiCell Laboratories or from other sources online. Previously, we obtained fibroblasts derived from dermal foreskin (catalog designation: *DF6-9-9T.B,* WiCell Laboratories).
b. The human iPS cells were grown and maintained as per the company's instructions.
c. For control cells, normal adult human dermal fibroblasts were procured from the American Type Culture Collection (ATCC) and cultured according to the supplier's instructions. We also isolated and grew standard TM cultures and used donated HUVEC cells (a gift from Dr. Nabil Alkayed, Oregon Health & Science University, Portland, Oregon) and embryoid bodies (EBs) we generated from iPSCs. Other control cell cultured lines may be necessary for the investigator's experiments.

Generation of Embryoid Bodies:

a) *First, prepare extracellular matrix (ECM) derived from TM cultures*:

1. Grow human TM primary cultures in 6-well plates for 7 days using a standard TM growth medium to produce an extensive ECM.
2. Remove the TM cells by treating the cultures with 0.1% saponin for 1 hour, followed by washing extensively with PBS.
3. The ECM is then incubated for several days in PBS prior to use. No cells should be visible after this time, but it is best to check the surface under a microscope to make sure all the TM cells have been removed.
4. The ECM plates may be wrapped tightly with parafilm and stored for several months frozen at -20 degrees C.

b*) Second, prepare the conditioned medium (CM):*

1. Prepare TM cell cultures in T-75 flasks using serum-free TM medium (DMEM high/low glucose medium-1:1, 1% antibiotic/anti-mycotic).
2. When the incubated TM cell cultures (37 degrees C and 5% CO_2**)** are confluent or nearly so, collect the conditioned medium (CM) after 48 hours. Centrifuge the CM at 15,000 xg for 15 minutes.
3. Use the TM cell-conditioned medium as a part of DiffMedium in the differentiation of the EBs below.
4. The CM may be stored in sterile tubes at -20 degrees C for several months until ready for use.

c) *To generate the embryoid bodies (EBs*) to be differentiated to TM-like iPSCs on a large scale, detach medium-sized undifferentiated iPS cell colonies from 6-well plates by treating them with Accutase (Innovative Cell Technologies) as suggested by the manufacturer.

1. This procedure will generate uniform small-size EBs.
2. Transfer the detached cells to AggreWell plates (Stem Cell Technologies) and add AggreWell medium (Stem Cell Technologies) for 24 hours.
3. Transfer the EBs from the AggreWell plates to 6-well plates and initiate differentiation (Fig. **1**).

Differentiation of EBs

a) Differentiation is initiated by culturing the EBs on TM-cell derived ECM in DiffMedium, concocted by a mixture of:

1. standard TM cell growth medium (25%):

a.) medium-glucose Dulbecco's Modified Essential Medium-[DMEM; Gibco], a 1:1 mix of high and low glucose media.
b.) 10% Fetal Bovine Serum [Hyclone/Thermo Scientific]
c.) 1% Antibiotic/Anti-mycotic [100X; Life Technologies]

1. AggreWell medium (50%) [Stem Cell Technologies]

CHAPTER 10

Protocols to Isolate and Characterize Ciliary Muscle Cells and Schlemm's Canal Cells and Detect Matrix Metalloproteases

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Abstract: Ocular hypertension and the ensuing glaucomatous optic neuropathy occur when the aqueous humor drainage system within the anterior chamber of the eye, trabecular meshwork (TM), and its associated Schlemm's canal (SC) system become clogged due to the abnormally deposited excess extracellular matrix components. A similar series of events plague the ciliary muscle (CM) bundles in the uveoscleral pathway for AQH drainage. In order to study pathological events and endogenous chemicals associated with the latter tissues, isolated cells of TM, SC, and CM have proven useful. This chapter provides a brief and simple description of protocols to 1) isolate and characterize ciliary smooth muscle cells, 2) isolate and characterize Schlemm's canal cells, and 3) measure enzyme activities and expression pattern of matrix metalloproteases (MMPs) secreted from the isolated ocular cells using zymography and Western blotting. These studies are important for the characterization of the biochemical pharmacology of the drugs that activate specific receptors and enzymes that mediate the aqueous humor outflow from the anterior chamber of the eye to lower the IOP.

Keywords: Ciliary muscle cells, Glaucoma, Intraocular pressure, MMPs, Zymography.

INTRODUCTION

Ocular hypertension (OHT) is a major risk factor associated with open-angle and closed-angle glaucoma. OHT or elevated intraocular pressure (IOP) develops when excess aqueous humor (AQH) accumulates in the anterior chamber of the eye due to the partial occlusion of the trabecular meshwork (TM) and the associated Schlemm's canal (SC) and the AQH drainage pathway between ciliary muscle (CM) bundles. In order to better understand the pathological factors and

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events involved in the aforementioned occlusive processes, studies conducted on isolated TM, SC, and CM cells are necessary. Furthermore, the potential mechanisms of action of potential IOP-lowering agents can be determined using such cell isolates. TM cell isolation is described in Chapter 8 of this book; therefore, the current chapter will focus on the other cell types.

Isolation and Characterization of Ciliary Smooth Muscle Cells

Ciliary smooth muscle cell cultures were established from post-mortem human eyes (donors age range, 45–89 years), and human tissue in this study conformed to the tenets of the Declaration of Helsinki. The human eyeballs should be obtained within 24-72 hours after death and kept moist in ice. The following procedures are recommended for the isolation of human (or animal) ciliary smooth muscle (SM) cells.

- Upon arrival of human eyes in the laboratory, ciliary muscles should be immediately dissected with the aid of a dissecting microscope under sterile conditions. Briefly, the eyes must be equatorially bisected and the posterior half should be removed. The anterior half of the eye can then be placed in a sterile Petri dish. The zonules can be cut and the lens removed.
- To remove the ciliary muscle, carefully insert one branch of fine forceps in the supraciliary or suprachoroidal space of the specimen and gently pull the ciliary muscle tissue away from the underlying sclera. This also allows detachment from the scleral spur. The ciliary muscle can be recognized as a broad, pale, and circular band, which should then be cleaned and cut into small pieces (1 to 2 mm) with a fine pair of scissors under the dissecting microscope.
- In order to isolate the ciliary SM cells, place the cut ciliary muscle pieces in DMEM-199 culture medium containing 2 mg/mL collagenase type IA, 10% FBS, and 50 μg/mL gentamicin in a sterile 25-50 ml plastic tube and incubate for 1 to 2 hours at 37°C with occasional gentle mixing/shaking. The major parts of the explants should be dispersible into single cells or groups of cells after this time.
- Centrifuge the contents of the tube at 200*g* for 10 minutes and discard the supernatant. Next, resuspend the pelleted cells in fresh DMEM-199 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B to help cells stabilize and the future cell cultures to be free of any potential contamination.
- A suspension of the cells from above should be incubated in 100 mm tissue culture Petri dishes for 1 hour at 37°C in a 5% CO_2 humidified incubator to allow the contaminating fibroblasts to attach to the bottom of the dish.
- After this time, the floating non-adherent cells (ciliary SM cells) can now be transferred to another Petri dish and incubated at 37°C in a 5% CO_2 humidified

incubator to allow them to attach and proliferate. As mentioned above, the DMEM-199 medium should contain 1% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B.

- Replace the culture medium after 3 days and check the morphology of the cells under the phase contrast microscope to ensure cells look healthy and uniform as much as possible (see Husain *et al*., 2002; Fig. **1**).
- When the ciliary SM cells are 80-90% confluent (within 3-5 days), these cells should then be passaged using 0.05% trypsin and 0.02% EDTA to detach the cells; the medium should be diluted with fresh medium to stop trypsin activity and centrifuged in appropriate tubes. The supernatant is aspirated, the cells are washed and spun down, the pellet is resuspended in a fresh growth medium, and the cells are replated using a split ratio of 1:4 (Husain *et al*., 2002).

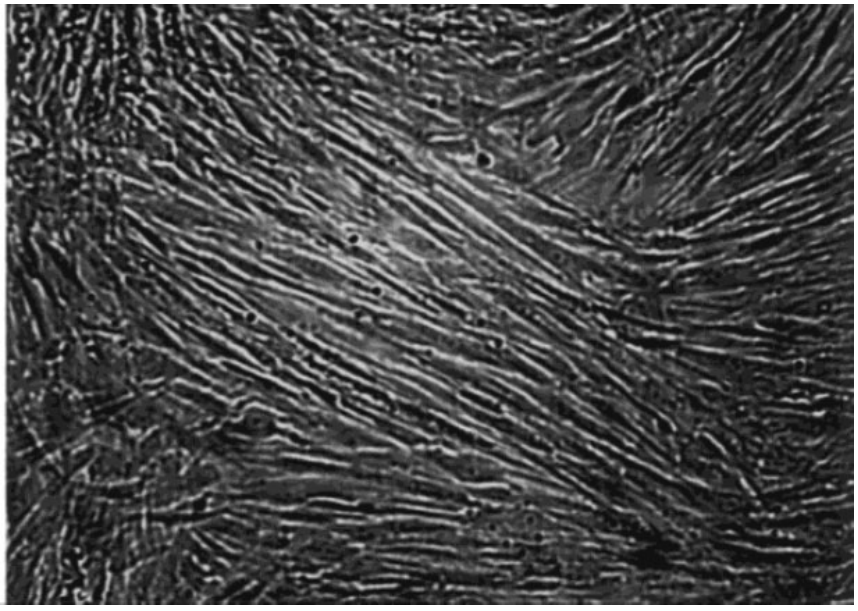

Fig. (1). Phase contrast image of normal human ciliary muscle (HCM) cells. HCM cells have typical hill-and-valley arrangement of growth as described and shown earlier. (Husain, Kaddour-Djebbar *et al.* 2002).

Characterization of Ciliary Smooth Muscle Cells by Immunocytochemistry

- In order to confirm that the isolated and cultured human ciliary SM cells are not contaminated with fibroblasts, characterize the cells using specific antibodies as described by Husain *et al*., (2002).
- Briefly, culture the cells to 70-80% confluency on sterile glass microscope slides with individual chambers and carefully fix them with ice-cold methanol for 15 minutes. After this time, carefully and gently rinse with phosphate-buffered saline (PBS) buffer 3 times with a 5-minute interval between each wash.

CHAPTER 11

A Protocol for Primary Culture of Non-Pigmented Ciliary Epithelium Cells

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Abstract: Here, we provide a methodology for routine primary culture of porcine non-pigmented ciliary epithelium (NPE), a cell type that plays a critical role in AH formation. Using fresh eyes from adult pigs, the dissection protocol allows us to obtain the entire ring of NPE as a ruffled sheet of cells attached to the vitreous. Dispersal of the cells for initial culture is made possible by treatment with collagenase and hyaluronidase. We describe the seeding density, time to confluence, and trypsinization protocol for NPE cells established in primary culture. Comparison of the freshly isolated and cultured NPE cells includes analysis of several proteins known to be characteristic of the NPE. This includes measuring the expression of xenobiotic transporters (PgP, BCRP, MRP4, MRP 2, MRP1), connexins (Cx-43 and Cx-50), sodium-hydrogen exchangers, and carbonic anhydrases. The diffusion barrier property of cultured NPE monolayers is also examined. Prior to the development of this technique in 2007, experts in the field did not believe it was feasible to culture and propagate NPE. Our success hinged on being able to seed freshly isolated NPE cells at a sufficiently high density. This, in turn, hinged upon being able to isolate a relatively large quantity of NPE cells, first by isolating the entire ciliary ring from each eye, then by dispersion of cells by enzyme treatment of the cell-to-vitreous body attachment.

Keywords: Ciliary body, Non-pigmented ciliary epithelium, Pigmented ciliary epithelium, Porcine eye, Primary culture.

INTRODUCTION

Glaucoma is a neurodegenerative eye disease in which the hallmark pathological lesion is the deterioration and death of ganglion cells, the neurons that transmit visual information from the retina to the brain. The underlying causes of glaucoma appear to be multifactorial and are poorly understood. However, the most common form of the disorder, primary open-angle glaucoma, is associated

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with abnormally high fluid pressure within the eye (ocular hypertension). Because lowering intraocular pressure (IOP) is the most effective way to reduce the rate of glaucomatous vision loss (Kass, Heuer *et al.* 2002), there is considerable interest in the dynamics of aqueous humor (AH) flow through the eye. Normal IOP reflects a perfect balance between aqueous humor formation and drainage, and high IOP commonly results from inadequate aqueous humor exit (Acott, Vranka *et al.* 2021). IOP-lowering medications either slow AH formation or improve drainage. The continuous flow of AH through the anterior chamber of the eye is critically important. It supplies nutrition to the avascular tissues of the eye, removes metabolic wastes, and establishes the IOP required for the structural rigidity and optical alignment of the eye.

Here, we describe a technique for the primary culture of non-pigmented ciliary epithelium (NPE), a cell type that plays a critical role in AH formation. The site of AH production is the ciliary body (CB), a ring-like structure situated between the retina and the iris root. The base of the CB contains the ciliary muscles, forms the ciliary ring, and connects to the choroid. Closest to the retina is a flattened zone of the CB, the *pars plana*, which is the anchor point for the Zonules of Zinn that hold the lens in place and keep it under some degree of tension (Bassnett 2021). However, most of the CB, the *pars plicata*, is an elaborate arrangement of highly vascularized radial projections called the ciliary processes (CP). The stroma and leaky capillary network inside each CP are covered by the double layer of ciliary epithelial cells, the NPE, and the pigmented epithelium (PE), which are connected by gap junctions at their apical surfaces (Shahidullah and Delamere 2014, Li, Shan *et al.* 2018). The NPE faces the aqueous humor, and the PE layer faces the stroma. AH secretion is a metabolically active process whereby fluid from the stroma passes across the PE-NPE bilayer into the posterior chamber of the eye (Edelman, Sachs *et al.* 1994, Civan and Macknight 2004).

NPE cells are specialized. They have the physiological characteristics and morphology of a fluid-transporting epithelium, including extensive invaginations in the basolateral membranes facing the AH, tight junctions between the apical membranes, abundant mitochondria, and well-developed rough endoplasmic reticulum (Tormey 1966, Schlötzer-Schrehardt, Müller *et al.* 1990, Wolosin, Chen *et al.* 1993). The PE and NPE coordinate as a functional unit to secrete AH (McLaughlin, Zellhuber-McMillan *et al.* 2004), and the NPE plays a particularly important role because it is the source of almost all the Na, K-ATPase activity that provides the driving force (Shahidullah, Wilson *et al.* 2003). Na, K-ATPase is highly enriched in NPE cells. The cells express multiple Na, K-ATPase isoforms at the basolateral surface, which faces the AH (Martin-Vasallo, Ghosh *et al.* 1989, Ghosh, Hernando *et al.* 1991, Coca-Prados, Fernández-Cabezudo *et al.* 1995, Shahidullah, Tamiya *et al.* 2007). In order to develop remedies to slow AH

secretion and lower intraocular pressure for the treatment of glaucoma, it would obviously be useful to understand the individual contribution of NPE and PE to AH formation. While there have been several studies in this area (Tormey 1966, Shi, Zamudio *et al.* 1996, Patil, Han *et al.* 2001, Do and Civan 2009), questions remain. Thus, establishing protocols for the primary culture of PE and NPE would be useful. It turns out that PE cells, which are thought to be developmentally related to retinal pigment epithelium (Coca-Prados, Fernández-Cabezudo *et al.* 1995), are comparatively easy to grow in primary culture (Helbig, Korbmacher *et al.* 1988, Helbig, Korbmacher *et al.* 1989, Coca-Prados, Ghosh *et al.* 1992, Bode, Hamel *et al.* 1993, Shahidullah and Wilson 1999, Fleisher, McGahan *et al.* 2000, Hochgesand, Dunn *et al.* 2001). In contrast, NPE cells are developmentally related to the neural retina (Coca-Prados, Fernández-Cabezudo *et al.* 1995), and obtaining primary cultures of NPE monolayers has proved to be difficult. For many years, it was believed that NPE cells could not be grown *in vitro*. However, after more than 10 years of trial and error, we were able to isolate NPE cells from the porcine eye and establish the cells in primary culture (Shahidullah and Delamere 2006), forming monolayers that retain many of the *in vivo* NPE characteristics (Shahidullah, Tamiya *et al.* 2007). In this chapter, we describe in detail the protocol for porcine eye dissection, isolation of NPE in bulk, and culturing the cells in monolayers.

EXPERIMENTAL PROTOCOLS

The use of animals in the following procedure was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona and complied with the Association for Research in Vision and Ophthalmology guidelines on the use of animals in research.

Preparation of the Porcine Eye for Dissection

Fresh eyes from young adult pigs (6-10 months; usual slaughter weight of 280 lbs. or more) were collected from a local abattoir and transported to the lab on ice. The eyes were enucleated from the carcasses along with reasonable remnants of extraocular muscles, avoiding undue pressure that might negatively affect internal structures. In the lab, the remnants of extraocular muscles and fat were trimmed away using a pair of surgical scissors and a pair of rat tooth forceps. The eyes were washed with Dulbecco's phosphate-buffered saline containing calcium and magnesium (Gibco™ 14040133) that also contained a high concentration of Gentamicin (400 µg/ml) (Sigma, Cat # G1397) and then placed in serum-free DMEM (Gibco, Cat # 12430-062) containing 200 µg/ml Gentamicin in a biological safety cabinet for subsequent dissection. The washing and storing of

CHAPTER 12

Anterior Segment Scleral Fibroblasts and Protocols for Ocular Cell Isolation and Characterization for Glaucoma Studies

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Abstract: Scleral fibroblasts (SF) are crucial in maintaining and remodeling the extracellular matrix of the sclera, which is vital for preserving the structural integrity and biomechanical properties of the eye. Investigating the behavior and characteristics of SF is essential for understanding the development of various ocular conditions. This chapter provides an overview of the protocols for isolating and characterizing SF, with a specific focus on their relevance to glaucoma research on the anterior segment.

The initial step in studying SF involves isolating them from either animal models or post-mortem human eyes. Sterile conditions are maintained during the dissection and enzymatic digestion of scleral tissue using collagenase or trypsin. The resulting cells are then cultured in an appropriate growth medium supplemented with serum and antibiotics. Immunocytochemistry is employed to characterize the isolated SF, and flow cytometry can be used to analyze cell-surface markers associated with the SF phenotype. Co-culture experiments provide valuable insights into the interactions between SF and other cell types relevant to glaucoma, such as retinal ganglion cells or trabecular meshwork cells. *In vitro* models can be developed to mimic glaucomatous conditions by subjecting SF to mechanical stress or cyclic stretching, enabling the study of their response to elevated intraocular pressure. Secretome analysis techniques, such as ELISA or multiplex immunoassays, can be employed to examine the factors secreted by SF cultures, providing insights into their role in modulating glaucoma-related processes. In conclusion, these protocols serve as a valuable resource for researchers investigating SF in the context of glaucoma research on the anterior segment.

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Keywords: Cell culture, Characterization, Cell proliferation, Co-culture, Extracellular matrix, Flow cytometry, Isolation, Immunocytochemistry, Scleral fibroblasts, Viability assays.

INTRODUCTION

Glaucoma is a progressive optic neuropathy characterized by the degeneration of retinal ganglion cells (RGCs) and corresponding visual field loss. It is one of the leading causes of irreversible blindness worldwide, affecting millions of individuals (Mélik PS *et al*., 2020). Despite extensive research, the underlying mechanisms of glaucoma pathogenesis remain incompletely understood (Razeghinejad R *et al*., 2020). However, recent studies have suggested the involvement of various ocular cell types, including scleral fibroblasts, in the development and progression of glaucoma (Wong, T. Y. *et al*., 2014; Downs JC., 2015; Pitha I, *et al*., 2018; Minnelli C *et al*., 2023).

The anterior segment of the eye plays a crucial role in maintaining ocular homeostasis and proper visual function. Among the various cell types present in the anterior segment, scleral fibroblasts have gained significant attention in recent years due to their involvement in the pathogenesis of glaucoma, a leading cause of irreversible blindness worldwide. Scleral fibroblasts are responsible for maintaining the structural integrity of the sclera and regulating extracellular matrix (ECM) remodeling, which can directly impact intraocular pressure (IOP) and optic nerve health (Soma, T., *et al*., 2017). They are responsible for synthesizing and remodeling the ECM, which provides mechanical support to the ocular tissues (Downs JC., 2015; Wang, W., *et al*., 2017). In glaucoma, alterations in scleral fibroblast behavior and ECM remodeling have been observed, leading to changes in scleral biomechanics and potentially contributing to the development of elevated IOP and retinal ganglion cells (RGC) degeneration (Agarwal R *et al*., 2019; Iomdina EN *et al*., 2020; Boote C. *et al*., 2020). An area of key importance is the ability of FP-receptor-class prostaglandins to release matrix metalloproteinases from SF to help digest ECM in the uveoscleral outflow pathway to lower IOP in the treatment of glaucoma (Weinreb et al., 2004; Sharif *et al*., 2023).

To better understand the molecular mechanisms underlying glaucoma and the involvement of scleral fibroblasts, it is essential to isolate and characterize these cells effectively. Several protocols have been developed to isolate ocular cells from the anterior segment, including the sclera. These protocols involve the enzymatic digestion of ocular tissues, followed by cell isolation and culture techniques tailored to the specific cell type of interest.

In this chapter, we will discuss the various protocols available for the isolation and characterization of scleral fibroblasts, focusing on the anterior segment of the eye. We will explore the different enzymatic digestion methods, the selection of appropriate culture media and conditions, and the characterization techniques commonly used to assess fibroblast phenotype, viability, proliferation, and ECM production. Additionally, we will highlight the importance of quality control measures to ensure the reliability and reproducibility of the isolated cells.

Understanding the molecular and cellular changes in scleral fibroblasts associated with glaucoma can provide valuable insights into the pathogenesis of the disease. This knowledge may lead to the identification of novel therapeutic targets for the prevention or treatment of glaucoma, as well as the development of tissue engineering strategies aimed at restoring scleral homeostasis and preserving visual function.

EXPERIMENTAL PROTOCOLS

The following procedures were performed in compliance with the guidelines of the Canadian Council on Animal Care for the Use of Experimental Animals and the Statement for the Use of Animals in Ophthalmic and Visual Research from the Association for Research in Vision and Ophthalmology (ARVO).

ISOLATION OF SCLERAL FIBROBLASTS

Materials

Enucleated eyes from human or animal donors

Dulbecco's modified Eagle's medium (DMEM)

Fetal bovine serum (FBS)

Penicillin-streptomycin solution

Trypsin-EDTA solution

Phosphate-buffered saline (PBS)

Sterile surgical instruments (scalpel, forceps, scissors)

70% ethanol

Tissue culture plates or dishes

Cell culture incubator (37°C, 5% CO_2)

CHAPTER 13

Protocols for Isolation and Characterization of Human Corneal Epithelial and Conjunctival Epithelial Cells

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Abstract: The eye is a specialized organ composed of many types of tissues and cells. Since the ocular surface is the first point of contact with light entering the eye, environmental airborne materials, and topically applied medications, it is important to understand the features of these cells and their physiopathology. Furthermore, such characteristics and receptor/enzyme/transporter profiles of the cells can serve as targets for drug discovery and development to treat such eye disorders and/or to show the potential risks of ocular irritation and inflammation associated with certain medications. This is particularly important with respect to corneal and conjunctival epithelial cells and mast cells, which are involved in the disease mechanisms associated with dry eye syndrome, ocular allergies, and ocular surface pain. The isolated cells can also be used to study the mechanisms of actions of certain drugs such as anti-histamines, mast cell stabilizers, and steroids. This chapter aims to provide protocols to isolate, propagate, and study cells obtained from human cadaveric donor tissues. Potential ways to immortalize human corneal epithelial cells will also be described.

Keywords: Conjunctival cells, Corneal epithelial cells, Human corneal cells, Mast cells, Protocols.

INTRODUCTION

The corneal epithelium is composed of multiple layers of cells that cooperatively work together to help maintain the transparency and overall integrity of the cornea. These cells protect the eye from airborne dust, pollen, fungal spores, bacteria, viruses, and radiation. These cells can actively participate in mediating allergic and inflammatory responses and the actions of the adjoining conjunctiva. Conjunctival mast cells are a type of immune cell found in the conjunctiva, which

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is the clear tissue that covers the white part of the eye and lines the inner surface of the eyelids. Mast cells are a crucial part of the immune system and play a role in various ocular surface disorders (Abelson and Schaefer, 1993; Cook *et al*., 1998). Histamine and other mediators released by mast cells trigger allergic responses such as itching, redness, and swelling of the conjunctiva, leading to a condition known as allergic conjunctivitis. Recent findings on the role of cytokines released by mast cells have heightened interest in the role of mast cells in allergic conditions (Abelson and Schaefer, 1993; Kahn *et al*., 1993; Bradding, 1996; Sharif *et al*., 1996; Cook *et al*., 1998; Sharif, 2020).

Described below are procedures to isolate, characterize, and immortalize cells derived from human ocular surface tissues, including human corneal epithelial cells, human conjunctival mast cells, and epithelial cells (Cook *et al*., 1998). The primary cells and immortalized cell line(s) can be used in mechanistic studies on ocular toxicity, irritation, and inflammation, and the latter provides a model to examine mechanistically the effect of mast cell-derived mediators on ocular surface epithelial cells and fibroblasts (Wiernas *et al*., 1998; Sharif *et al*., 1998a,b; Offord *et al*., 1999; Sharif and Wiernas, 2010). Cells isolated from the human ocular surface tissues can be invaluable in exploring mechanisms of action of therapeutic drugs to treat diseases such as dry eye, allergic reactions, and pain associated with the latter or that induced by certain medicines, foreign objects, airborne pollutants, bacteria, and viruses (Abelson and Schafer, 1993; Sharif *et al*., 1997; Yanni *et al*., 1999; Sharif, 2020). Such cellular characterization and utility can lead to the discovery of new drugs to treat ocular surface disorders.

EXPERIMENTAL PROTOCOLS

Generally, it is recommended that the described procedures below be performed in a laminar flow-hood under sterile conditions using sterilized reagents and instruments as supplied by the manufacturers. Instructions provided should be followed to ensure the success of the experiments. Researchers are encouraged to consult original cited papers for possible additional guidance and to confirm the conditions and study procedures. Some key publications to consult include the following:

Sharif *et al*., 1997; Yanni *et al*., 1999; Offord *et al*., 1999; Sharif and Wiernas, 2010.

PREPARATION, CHARACTERIZATION, AND IMMORTALIZATION OF PRIMARY CULTURES OF HUMAN CORNEAL EPITHELIAL CELLS

Cell Isolation and Characterization

- Obtain human donor eyes after death (ensure proper consent has been obtained). Dissect the cornea aseptically and rinse in phosphate-buffered saline (PBS).
- Treat with dispase at 10U/ml in 50% Hanks buffered salts and keratinocyte growth medium (KGM) at 4°C for 24-48 hrs (prepare KGM by adding 30µg/ml bovine pituitary extract, 0.5µg/ml hydrocortisone, 5µg/ml insulin, 10µg/ml transferrin, 10ng/ml murine epidermal growth factor, 0.05µg/ml amphotericin B, and 50µg/ml gentamicin to keratinocyte basal medium (KBM) that contains 0.15 mM calcium chloride. KBM, growth factors additives, and antibiotics can be purchased from Clonetics, San Deigo, CA, or Biofluids, Rockville, MD)
- Remove epithelium after incubation by gently sweeping across the tissue with a sharp scalpel blade (if possible sterile).
- To generate individual cells, triturate the tissue and then wash in Dulbecco's modified Eagles medium that contains 10% fetal bovine serum by using low-speed centrifugation-resuspension procedure.
- Resuspend the cell pellet in KGM and plate it into T25 flasks that have been previously coated with 0.1% gelatin type A (can be purchased from Sigma; Fluka Chemie AG, Buchs, Switzerland)
- Incubate at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 and change the medium after 24hrs and then every 2 days thereafter.

Cell Immortalization

- Grow primary cultures of human corneal epithelial (P-CEPI) cells to subconfluence in KGM.
- Infect the cells 8 days after isolation with a high-titer recombinant retroviral vector pZIPSVU19, which contains the Bgll-Hpal frag-ment of SV40 T antigen and the neomycin selection marker from Moloney virus (details seen in Mace *et al*., 1996; Pfeifer *et al*., 1995; Offord *et al*., 1999). The process is briefly described in the next few steps.
- Generate a high-titer virus through transfection of pZIPSVU19 into ecotropic and amphotropic packaging cell lines Psi2 and PA137, respectively, followed by coculture of the two cell lines to allow "ping-pong " infection to produce a high-titer virus.
- Infect the P-CEPI cells with 105 to 107 CFU recombinant SV40 virus in the presence of 8 /ng/ml polybrene for 2 hours. (Because of a truncated origin of replication, no SV40 can be subsequently produced in the immortalized cell

OCULAR CELL-BASED *IN VITRO* ASSAYS TO DETERMINE PHARMACOLOGICAL PROPERTIES OF KEY RECEPTORS/ENZYMES/CHANNELS AND FOR SCREENING COMPOUNDS

CHAPTER 14

Overview and Examples of Drug Screening in Cell- and Tissue-Based Assay Systems: Screening Funnels for *In Vitro* and *In Vivo* Studies

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Abstract: Chronically elevated intraocular pressure (IOP) is a hallmark of open-angle glaucoma, the most prevalent form of this neurodegenerative eye disease around the world. Although a number of drugs have been approved for lowering and controlling IOP, there continues to be an unmet medical need for superior therapeutics that exhibit better pharmacodynamics and can overcome the significant compliance issues associated with current medicines. As such, it is necessary to continue drug discovery research using both *in vitro* and *in vivo* screening methods. In terms of the former, several cell- and tissue-based assay systems have been developed and implemented in compound screening paradigms that rely on a variety of readouts to assess receptor binding affinity, functional potency, and relative selectivity. In the current chapter, an overview of many functional assay systems using cultured cells or intact tissues derived from ocular tissues involved in ocular hypertension will be presented.

Keywords: CAMP, CGMP, Enzymes, Gaseous transmitters, Inositol phosphates, Receptors.

INTRODUCTION

Glaucoma is an insidious thief of sight, being an asymptomatic neurodegenerative retinal/optic nerve disease that affects millions of people worldwide. One form of glaucoma in which a strong association has been built between the etiology of the disease process and chronically elevated intraocular pressure (IOP) is open-angle glaucoma (OAG) (Weinreb *et al*., 2014; Sharif, 2018, 2020, 2021; Sharif *et al*., 2023). Even though many drugs have been introduced into the medical manage-

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ment of OAG and ocular hypertension (OHT) (Bucolo *et al*., 2018; Sharif, 2021), they have some serious side effects that limit their life-long use to lower and control IOP. These untoward issues have a major effect on the patient's compliance to administer the prescribed eyedrop medications. Thus, there is still an unmet medical need to find and market new drugs that have a better therapeutic index than the existing treatment modalities. In order to screen and characterize new potential drug candidates, a number of *in vitro* assays need to be utilized using isolated cells from the anterior chamber of the eye tissues involved in regulating IOP, such as trabecular meshwork (TM), ciliary muscle (CM), ciliary epithelial cells (CEPI), and perhaps even Schlemm's canal cells, although the latter are more difficult to obtain (Sharif, 2018; Ellis *et al*., 2010). Data from such cost-effective assay systems allows to profile many compounds and progress them, based on their rank orders of receptor affinities and functional potencies, to *in vivo* studies, which are more expensive and labor-intensive when implemented in screening funnel formats (Sharif, 2018; Figs. **1A** and **1B**).

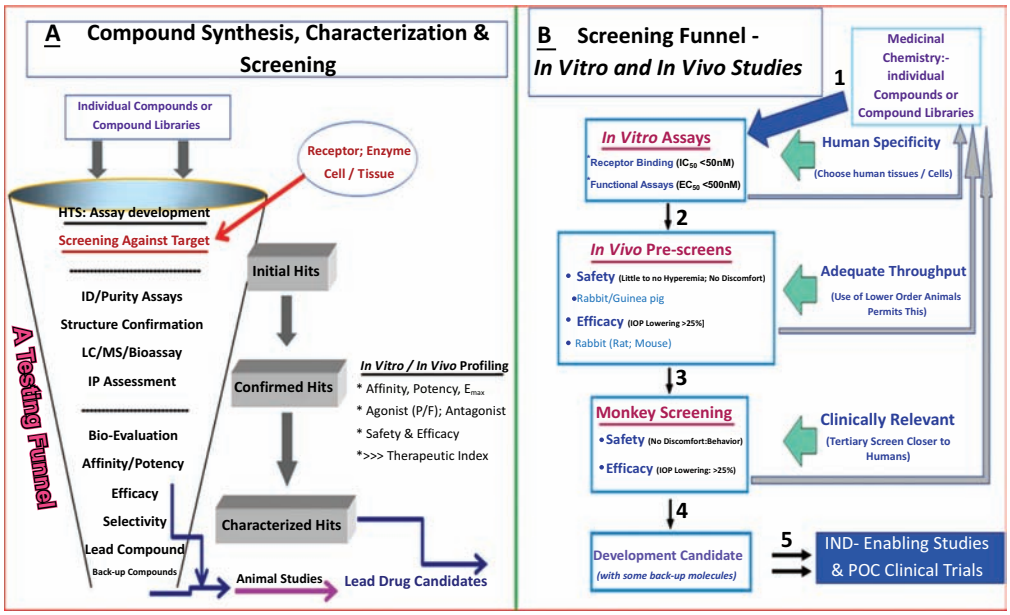

Fig. (1). Screening funnels with stage-gate parameter definition in cellular and *in vivo* studies.

Neurotransmitters, hormones, steroids, and a host of cyto- and chemokines produce their effects via specific receptors, ion channels, and transporters located in cell membranes of tissues and on intracellular organelles. Activation of transmembrane components of these proteins generates intracellular second messengers such as cAMP, cGMP, inositol phosphates, diacylglycerol, Ca^{2+}, and gaseous transmitters such as nitric oxide and hydrogen sulfide that modulate the

activity of cytoplasmic proteins, lipids and other cellular components via phosphorylation/dephosphorylation, acetylation, and glycation (see Sharif, 2018; 2021; Fig. **2**). Active modulation of genetic machinery via transcription factors, various chaperones, and secretome/exosome-mediated release of growth factors and microRNAs is also very important in cellular communication, as well as in providing intervention points to mitigate diseases associated with these molecules. The direct transfer of many of the aforementioned chemicals between neighboring cells can also occur via nanotunnels, and this represents a novel mechanism of cellular communication. For now, this chapter outlines methods used to ascertain the functional activity (potency and efficacy [intrinsic activity]) of test compounds in cell- and tissue-based assay systems using second messengers and cell/ tissue contraction/relaxation readouts in a screening funnel arrangement (Sharif, 2018, 2020, 2021, 2023; Figs. **1** & **2**). Those compounds that meet or exceed the *in vitro* criteria and requirements in terms of receptor/enzyme/ion-channel/transporter affinity, functional potency, and intrinsic activity can then be tested in a series of *in vivo* models. The animal-based studies encompass testing for ocular irritation and overall ocular safety by audio and visual observations (*e.g.*, in guinea pigs and rabbits) and for *in vivo* efficacy in terms of IOP lowering in rodents/rabbits/dogs/monkeys using the criteria set to progress from lower phylogenetic animals to the higher ones in order to reduce cost and labor-intensiveness (Fig. **1B**).

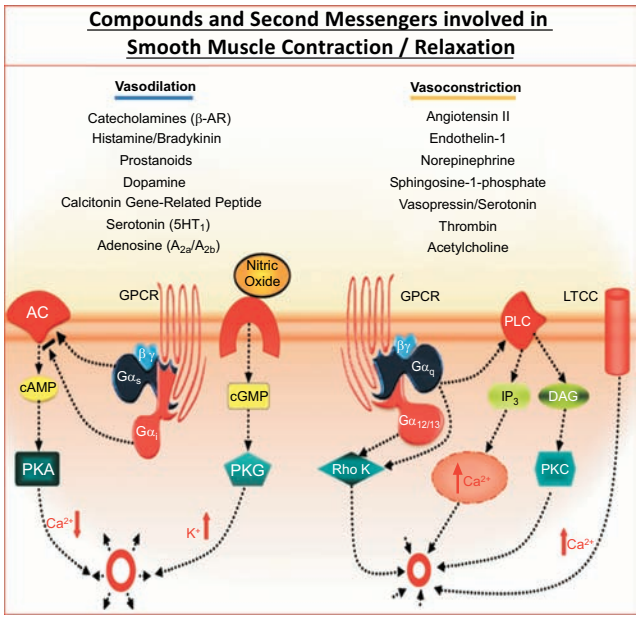

Fig. (2). Examples of neurotransmitters and drugs that can contract or relax the smooth muscle of blood vessels and other tissues.

CHAPTER 15

Protocol for Studying Ocular Cell Relaxation / Contraction by Cell Impedance

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Abstract: Pharmacological control of cellular contractility is a validated mechanism for the modulation of aqueous humor outflow and, thus, modulation of intraocular pressure (IOP). Label-free cellular impedance assays monitor the change in electron flow in response to environmental conditions, allowing the detection of responses to a diverse number of cellular stimuli, including the administration of biological or pharmacological agents that affect the actin cytoskeleton. The impedance assay has several differentiating factors compared to other cell-based assays, including the advantage of time-resolved dynamic measurements from living adherent cells. Numerous reports have described the utility of impedance assays in the characterization of the mechanism of action of individual ligands or small molecules. In addition, these assays are applied in screening campaigns designed to identify new pathways with phenotypes of interest. This chapter describes protocols and considerations for designing, validating, executing, and analyzing cellular impedance assays for the purpose of evaluating effects on cell contractility. The focus here is aimed at cells of the conventional aqueous outflow pathway, but the principles are broadly applicable outside of this field.

Keywords: Cell culture, Contractility, Intraocular pressure, Impedance, Relaxation, Screening.

INTRODUCTION

Cell-based electrical impedance is assessed by plating adherent cells on specialized culture vessels with gold electrodes and applying a continuous current at various frequencies. Cell membranes impede the applied electrical current moving from the electrode into the cell media, and the magnitude of the impedance can be quantified and represented mathematically. This enables indirect measurement of the state of cell morphology, which is a dynamic pheno-

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type of cellular response to the environment. Owing to the non-invasive and real-time nature of the measurements, numerous applications for the technology have been described, including assessment of cytotoxicity (Xing *et al*., 2005), cell adhesion and migration (Scrace *et al*., 2013), characterization of GPCR agonists and antagonists (Doijen *et al*., 2019), assessment of cell-cell junctions and barrier function (Benson *et al*., 2013), and screening of compound libraries for a variety of readouts (Thieulent *et al*., 2020, Vollmer *et al*., 2020). Of note, many of the above phenotypes and readouts are dependent on the state and behavior of the actin cytoskeleton.

A number of approved pharmacological treatments that lower IOP exert their effects through regulation of cell contractility and the actin cytoskeleton, including the Rho kinase inhibitors (Tanna and Johnson, 2018). Taken together, cytoskeletal modulation has been validated as an effective IOP-lowering mechanism. In the pursuit of additional compounds and targets that regulate the cytoskeleton, impedance platforms have been used to evaluate effects of numerous cell contractile and relaxing agents, as well as to understand the effects of Wnt signaling and cadherins (Stamer *et al*., 2010, Ramachandran *et al*., 2011a, Ramachandran *et al*., 2011b, Wang *et al*., 2013, Cavet *et al*., 2015, Webber *et al*., 2018).

The purpose of this chapter is to provide key experimental considerations and a framework for executing and interpreting cellular impedance assays. Two major commercial systems are available: the xCELLigence Real-Time Cell Analysis platform (xCELLigence RTCA, Agilent) and the Electric Cell-substrate Impedance Sensing (ECIS) platform (Applied Biophysics). Although experiments can be performed with manual manipulation, the quality, reproducibility, and scale of data obtained can be improved through the use of automated liquid handling instruments where possible. High-throughput screening is attainable with impedance platforms, as evidenced by our recent report describing a screen of a small library of ~4000 compounds over a few weeks, using a 384-well format and with multiple compound concentrations and replicates (Vollmer *et al*., 2020). The methods described here were developed using the xCelligence real-time cell analysis (RTCA) high-throughput screening (HTS) platform (Agilent Technologies Inc., California), but conceptually are broadly applicable to other platform types.

CELL SELECTION

For high-throughput compound screening, we recommend selecting immortalized cell lines derived from the tissue of interest (*e.g.*, Normal trabecular meshwork 5 (NTM5), an SV40-immortalized cell line generated at Alcon (Texas, USA) prior

to the acquisition of Alcon by Novartis in 2011). The use of primary cells is limiting for high-throughput screens with hundreds or thousands of compounds due to scarcity of material, potential for donor-to-donor variability, cell-type heterogeneity, and passage number limitations. Cell behavior may drift with increased passaging, so we would advise characterizing cells and ensuring maintenance of assay windows with passage number. However, smaller-scale screens and orthogonal confirmation studies can be carried out using primary cells derived from multiple donors. Primary TM cells should be validated for identity by assessing Myocilin protein production *via* a Western blot of whole cell lysates and conditioned media following prolonged (3 – 7 day) steroid treatment, per community recommendations (Keller *et al*., 2018). Ciliary muscle cells and Schlemm's Canal or surrogate commercially available endothelial cells such as human umbilical vein endothelial cells (HUVEC) may also be used and should be characterized according to current best principles (Tamm *et al*., 1991; Perkumas and Stamer, 2012).

LIQUID HANDLING AND AUTOMATION

RTCA HTS instruments use 384-well gold-electrode coated microplates; the control unit for this model has 4 ports, which enables connection of up to 4 instruments that can be operated simultaneously and independently. There are many high-throughput liquid handlers that can accommodate 384-well cell dispensing, plate-washing, and compound addition; we recommend that users consider taking advantage of automation where possible to reduce the variability in volumes dispensed or aspirated as well as the potential for human error while enabling swift administration of treatments. Overall, experiment quality will be significantly improved through the use of robotic liquid handling. We understand that not all laboratories will have access to liquid handling instruments, so as an alternative, the use of manual or electronic multi-channel pipettes is possible. While single-channel pipettes may be used, readers are advised that this may contribute to higher assay variability and diminished reproducibility. Impedance instruments with a lower throughput are also available, such as the ECIS platform, and can also be considered.

INSTRUMENT SETTINGS

Impedance instruments may have a variety of frequency settings, which affect the types of measurements that can be inferred, such as cell-cell contacts or cell density (Doijen *et al*., 2019). While the RTCA platform uses a fixed frequency (10,000 Hz) coupled with disposable plates, ECIS platforms enable the use of multiple frequencies ranging from 100 to 64,000 Hz. Responses to stimuli may vary according to the frequency selected, so method development should be

CHAPTER 16

Protocols for Studying Ocular Cell Volume Changes in Aqueous Humor Outflow Regulating Cells *In Vitro*

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Abstract: This chapter describes a protocol for measuring trabecular meshwork and Schlemm's cell volume with different experimental conditions over time, utilizing fluorescence and confocal microscopic methods. A fluorescence probe is applied to the cells, and a confocal microscope captures pixeled, z-stacked images with 8 bits of resolution. The images are converted from 8-bit to 1-bit or binary. Threshold values are determined using fluorescent latex beads of known diameter, and cell volume is analyzed. ImageJ software is used to calculate the number of voxels in the region of interest in the image stack. This methodology is of interest, as the outflow of aqueous humor is actively controlled by cellular and molecular mechanisms in the aqueous humor outflow pathway that includes the trabecular meshwork and Schlemm's canal. Several cellular mechanisms have been characterized by which changes in aqueous humor outflow can occur, including changes in the trabecular meshwork and Schlemm's canal cell volume.

Keywords: Cell volume, Fluorescence probe, Ocular hypertension, Schlemm's canal, Trabecular meshwork.

INTRODUCTION

Elevated intraocular pressure (IOP) places patients at an increased risk for developing visual field loss in the progressive blinding disease, glaucoma. IOP results from the balance between aqueous humor secretion by the ciliary processes and its outflow through the trabecular meshwork and Schlemm's canal (SC) within the anterior chamber of the eye. Major routes for the outflow of aqueous humor are the trabecular meshwork comprising of uveal and corneoscleral tra-

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becular meshwork (TM) and the juxtacanalicular cells (JCT-trabecular meshwork), in conjunction with the Schlemm's canal (SC), and also the uveoscleral pathway that occurs across the ciliary muscle (CM) through small spaces between CM bundles (Lee *et al*., 1982; Kaufman *et al*., 1984; Krupin *et al*., 1995). An imbalance of aqueous humor secretion and outflow will yield high pressures within the eye that can damage the retina and the optic nerve and thus cause loss of vision.

Once thought to be a passive process, the outflow of aqueous humor is now known to be actively controlled by the cells in the aqueous humor outflow pathways. Several cellular mechanisms have been characterized by which changes in aqueous humor outflow can occur. These include TM contractility and cell volume changes, CM contractility, alterations of the extracellular matrix in the TM, and formation of pores and vacuoles (Fig. **1**) in the inner wall of the SC (Banerjee *et al*., 2017; Mitchell *et al*., 2002; Soto *et al*., 2004; O'Donnell *et al*., 1995; Al-Aswad *et al*., 1999; Stumpff *et al*., 2000; Bradley *et al*., 1998; Grierson *et al*., 1998).

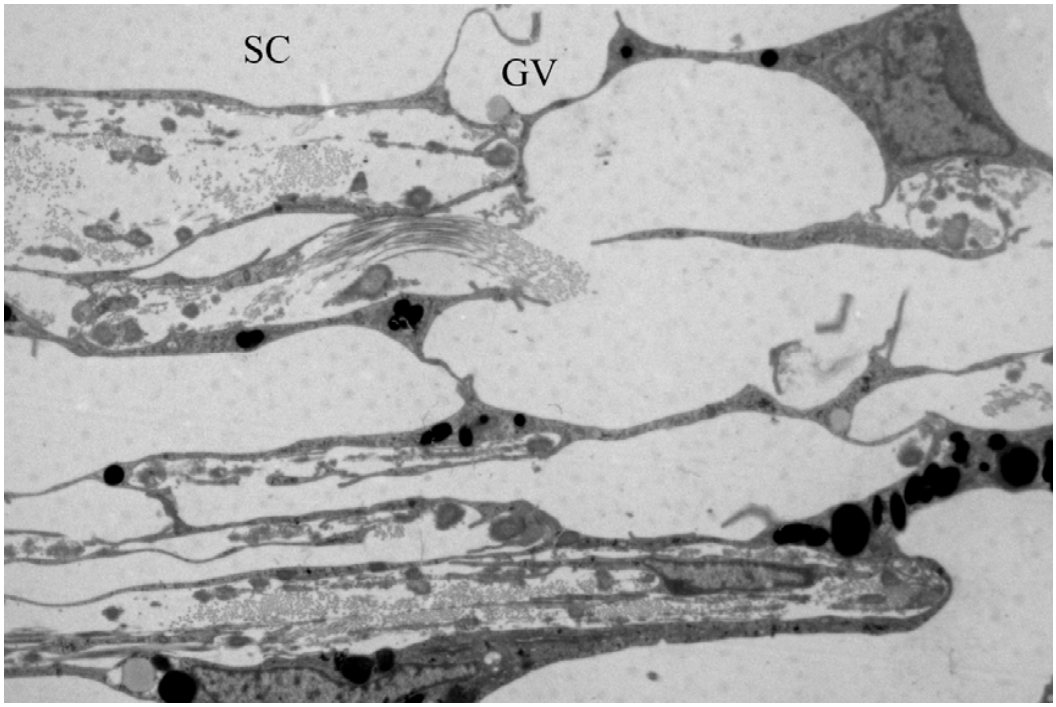

Fig. (1). Transmission electron micrograph of the aqueous humor pathway comprising the trabecular meshwork, JCT region, and Schlemm's canal (SC), showing giant vacuoles (GV) in the SC region. Image generated in the Ellis Lab.

Cell volume changes in the aqueous humor outflow pathway are of interest as they have been correlated with changes in the aqueous humor outflow facility. Studies demonstrate that a hypotonic or hypertonic challenge to a perfused eye anterior segment will decrease or increase the outflow facility respectively (Al-Aswad *et al*., 1999). To begin to understand mechanisms by which changes in TM and SC cell volume would affect aqueous humor drainage requires an understanding of the architecture of the aqueous humor outflow pathway (Fig. **1**). The TM is composed of interwoven collagen-covered elastin "beams". Trabecular endothelial cells (TM cells) line the outflow pathway and have been suggested to perform a number of functions, including phagocytosis (Polansky *et al*., 1984) and extracellular matrix (ECM) turnover (Alexander *et al*., 1991). As aqueous humor flows deeper into the TM towards the SC, the open spaces available for fluid to flow through decrease. As the spaces through which fluid flows narrow, the volume of the TM cells lining these openings would begin to exert more influence over the outflow facility. Increasing or decreasing the volume of these cells would, therefore, decrease or increase the rate by which fluid exits the eye, respectively.

Over the years of ocular cell volume regulation studies, several methodologies have been utilized to measure cell volumes. They include electronic cell sizing of suspended TM cells using a Coulter counter (O'Donnell *et al*., 1995; Li *et al*., 2011; Banerjee *et al*., 2017) and radioisotopes [^{14}C]urea and [^{14}C]sucrose as markers of cell volume of total intracellular and extracellular space(O'Donnell *et al*., 1995). While these are widely accepted methodologies, there are limitations on their uses. For example, electronic cell sizing utilizes non-adherent cells in suspension that have been treated with trypsin, a potential cell stressor and damaging enzyme. Additionally, the status of individual cells cannot be monitored during a given experimental run. This chapter describes a protocol that allows the measurement of cell volume of TM and SC cells that may be exposed to different experimental conditions over time using fluorescence and confocal microscopy (Mitchell *et al*., 2002; Bush *et al*., 2003 and 2005; Dismuke *et al*., 2008; Ellis *et al*., 2010)

EXPERIMENTAL PROTOCOLS

Cell Culture

Background

This section describes the protocol for the preparation of previously isolated and characterized primary cultured TM and SC cells for use in measuring cell volume (Fig. **2** and Table **1**) (Ellis *et al*., 2010).

CHAPTER 17

Protocols for Studying Aquaporin Function Related to Ocular Hypertension *In Vitro*

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Abstract: Aquaporins (AQPs) are a large family of membrane-bound water channels that facilitate water and small solute transport and have important physiological functions in all cells. At least thirteen types of aquaporins exist that exhibit a high level of phylogenetic homology, and many are present in ocular cells. Mouse knockout studies have demonstrated that AQPs play an important function in aqueous humor (AQH) dynamics in the anterior chamber cells and also have a role in retinal function. In contrast to the rich literature on aquaporin function, very little is known about how to assess water movement mediated by AQPs in their native environment, such as across ciliary epithelial cell membranes where AQH is manufactured. In part, the reason could be the lack of appropriate procedures for studying water movement across ciliary epithelial layers. This chapter describes methods to measure AQP function *in vitro* using ciliary epithelial cells, including a high-throughput screening assay method. In addition, a procedure to determine aquaporin function in its native environment is also presented.

Keywords: Aquaporins, Ciliary epithelium, Eye, Fluid transport, Glaucoma, Homeostasis, Intraocular pressure, Protocol, Retina.

INTRODUCTION

Aquaporins (AQPs) are a family of membrane channel proteins that transport water and small molecule solutes across cell membranes and possess important roles in physiological functions (Beitz and Schultz 1999; Agre, King *et al.* 2002; Verkman 2003, Patil; Wang *et al.* 2018,; Allegra, Cicero *et al.* 2022; Schey, Gletten *et al.* 2022; Ximenes-da-Silva, Capra *et al.* 2022; Bhend, Kempuraj *et al.* 2023; Bill 2023, Smith and Stroka 2023; Wang, Xu *et al.* 2023). Accordingly, AQPs play critical roles in a multitude of normal physiological and pathological functions and thus represent targets for drug discovery to modulate their activities. Selective inhibition of AQP can provide new approaches for treating many disor-

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ders, including ocular hypertension/glaucoma, corneal and macular edema, brain swelling associated with a stroke, *etc.* At least 12 types of AQPs prevail in mammalian tissues (Table **1**), and several members are present in the eye. AQP1, which is a water-selective channel, is present in the cornea, ciliary body, trabecular meshwork, retina, and micro-vessels of the choroid, regulating fluid homeostasis (Patil, Saito *et al.* 1997; Verkman 2003). Genetic knockout studies on mice have shown that AQP1 plays important functions such as in AQH dynamics and thus in intraocular pressure (IOP) regulation and retinal angiogenesis (Li, Patil *et al.* 2002; Zhang, Vetrivel *et al.* 2002). Ciliary epithelium, which is composed of the proximal (or outer) pigmented epithelium (PE) and the distal (or inner) non-pigmented epithelium (NPE), produces and secretes AQH (Fig. (**1**). These ciliary epithelial cells have been isolated and cultured and characterized for the presence of several ion transporters and receptors (Wax and Patil 1994; Mukhopadhyay, Geoghegan *et al.* 1997; Ortego and Coca-Prados 1999). It is known that AQH secretion is driven by the transport of electrolytes from the plasma into the posterior chamber of the eye, followed by the rapid movement of water and key solutes. In contrast to the rich information available for electrolyte movements, less is known about water movement across ciliary epithelial cell membranes *in situ* or in isolated cells.

Table 1. Listing of mammalian aquaporins.

Aquaporin	Size	Permeability	Distribution
AQP0	26 kD	*Water (poor)*	Lens
AQP1	28 kD	Water (ions?)	RBCs, Kidney, eye, brain
AQP2	29 kD	Water	Kidney
AQP3	31 kD	Water, glycerol, urea	Kidney, red blood cells, eye
AQP4	32 & 34 kD	Water (Hg insensitive)	Brain, eye, kidney, lung, ear
AQP5	29 kD	Water	Salivary gland, eye, lung
AQP6	28-30 kD	*Water (poor)*	Kidney
AQP7	26 kD	Water, glycerol	Adipose tissue, testis, kidney
AQP8	27 kD	Water	Testis, pancreas, liver
AQP9	32 kD	Water, glycerol, urea	Liver, testis, brain
AQP10	28 kD	*Water (poor),* glycerol	Small intestine
AQP11	30 kD	Water?	Liver, testis, kidney
AQP12	31 kD	Water?	Pancreas

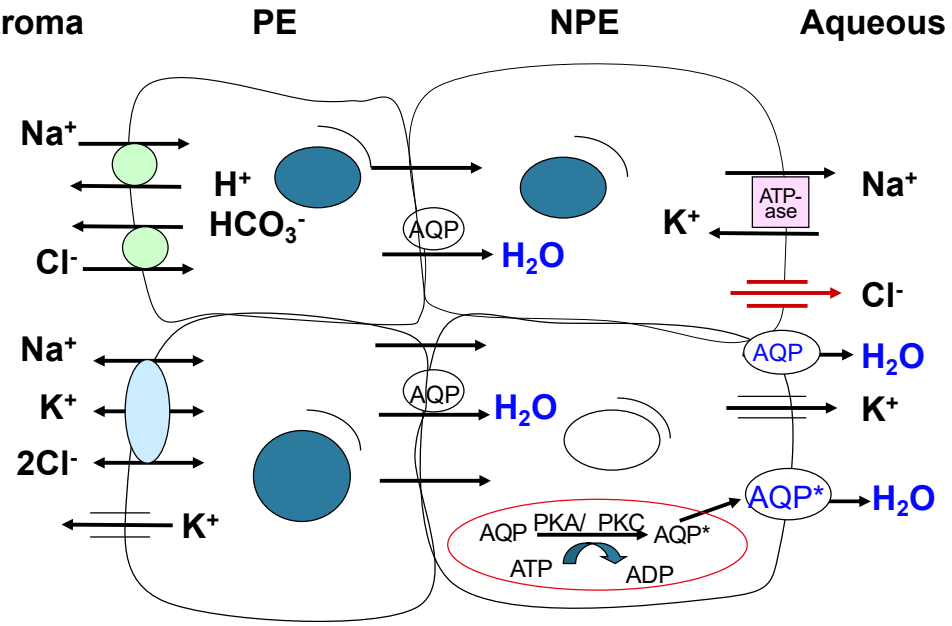

Fig. (1). Role of AQP1 in AQH production and secretion.

This chapter describes methods to measure AQP function *in vitro,* including a high-throughput screening assay, by capturing and analyzing the fluorescence dequenching of entrapped calcein in a confluent layer of AQP-expressing cells following exposure to a hypotonic shock. A high-throughput screening (HTS) assay method was successfully utilized to screen a library of novel compounds to identify potential AQP1 inhibitors/modulators (Patil, Xu *et al.* 2016). In addition, a procedure to assess AQP function in its native environment using proteoliposomes reconstituted with purified AQP1 is described below. These *in vitro* protocols represent valuable tools in defining the role of AQPs in normal physiological and pathological states. These methods can be used to find novel AQP1 inhibitors/modulators to treat many eye disorders, including ocular hypertension/glaucoma and edematous corneal and macular diseases.

Aquaporins Function in Native Cells Expressing AQP.

The *in vitro* method described below utilizes human non-pigmented ciliary epithelial (NPE) cells in culture to determine the function of aquaporin-1 in fluid transport by ciliary epithelium responsible for the secretion of aqueous humor into the posterior chamber of the eye.

CHAPTER 18

Measuring Elastic Moduli of Human Trabecular Meshwork Tissues, Cells, or Extracellular Matrices

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Abstract: Exploration of the mechanical properties of biological tissues has sparked a growing interest in the mechanobiology of various ocular diseases. There is an increasing recognition that the change in mechanical attributes (of cells/tissues) may reflect disease states or may be a viable endpoint to assess cell/tissue function. As such, mechanobiological investigations often rely on developing substrates with properties mimicking the mechanical properties of the choice of cell/tissue, either in homeostasis or in disease. Variability in methods of mechanical characterization has resulted in confounding reports that subsequently impact reproducibility in science and the development of appropriate scientific hypotheses. Here, we describe a protocol for measuring the elastic moduli of cells, matrices, and tissues relevant to the outflow pathway. We discuss factors to be considered while selecting cantilevers and preparing samples for measurements and provide detailed instructions for calibration of cantilevers, collection of force-displacement curves, and a workflow for how to analyze the data and determine elastic modulus. Although the protocol is intended for the cells/tissues of the iridocorneal angle, readers may apply or adapt this protocol for other relevant tissues.

Keywords: Atomic force microscopy, Elastic modulus, Glaucoma, Indentation, Primary cells, Trabecular meshwork.

INTRODUCTION

Biophysical stimuli – whether mechanical (such as pressure, stretch, compression, or shear), topographical (ordered or random topology), or non-biochemical soluble and insoluble signals (oxygen-tension, pH, or electric field) – represent an integral part of the cellular microenvironment and tissue functionality. Indeed, a growing body of research has demonstrated the profound impact of biophysics on tissue development, homeostasis, and disease. All the while, it is important to place them in the larger context of tissue function, which includes biochemical

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and genetic factors. A fundamental characteristic exhibited by all biological materials is their innate biomechanical property that drives and partakes critically in their intrinsic structure-function relationship. Biomechanical properties of tissues are defined by their constitutive elements: cells and their extracellular matrix (ECM). Cellular biomechanics have become recognized as important contributors to cell and tissue (dys)function essentially due to the changes in cytoskeletal dynamics that may be governed by phenotypic processes such as migration and differentiation, as well as remodeling of the extracellular matrix or response to drugs. Cells themselves are intrinsically capable of both sensing and exerting physical forces in a context-dependent manner(Jahed *et al.* 2014; Kaminski *et al.* 2014; Martino *et al.* 2018). For example, forces exerted by cells on their microenvironment allow for matrix remodeling after injury or in response to wound repair. Similarly, changes in matrix stiffness can influence the amplitude of force transmitted across cell-adhesion sites and subsequently to the cytoskeleton and nucleus, thereby influencing nuclear shape and gene expression (Dahl *et al.* 2008; Jaalouk and Lammerding 2009). This bidirectional ability to sense and respond to physical forces helps maintain homeostasis. It is thus conceivable that if a cell misinterprets and misprocesses these biophysical stimuli, the consequences on cell fate decisions or transcriptional regulation of genes and subsequent post-translational modifications of proteins (including but not limited to ECM) may all contribute to pathology. Untangling causal relationships between microenvironment and cellular behavior requires experimentation that carefully matches both the biochemical and biophysical environment of normal and diseased tissues. Thus, there have been several attempts to quantify the biomechanical attributes of several tissues (liver, neural, musculoskeletal, dermal, ocular, *etc.*), cells, and ECM in homeostasis and pathology.

In the eye, approximately 75% of all aqueous humor flows through the trabecular meshwork (TM) and Schlemm's canal (SC) (Toris *et al.* 1999; Toris *et al.* 2002). The site of major resistance to this outflow is thought to reside within the cribriform region of the meshwork, the area around the juxtracanalicular region (JCT) of the meshwork, and the inner wall cells of the SC (Johnson *et al.* 1992; Tamm 2009; Tamm 2010; Tamm 2015; Tamm and Fuchshofer 2007). Whilst there have been several debates on ascertaining the relative contributions of JCT/TM and cells of the inner wall SC to outflow resistance, it is commonly accepted that ECM at the meshwork, particularly in the JCT, is a major contributor to the resistance. In addition to lending resistance to outflow, constituents of the JCT, trabecular lamellae, and ECM components act as load-bearing structures that contribute to innate mechanical properties of the tissue. Ascertaining the mechanical properties of tissues is inherently difficult due to tissue inaccessibility. A wide range of mechanical properties and methods available for measurement are largely context-dependent. Indeed, mechanical

characterization of the TM has been performed either in isolation or in a combination of advanced optical imaging, indentation and tensile methods, acoustic force elastography, and finite element modeling (Ai *et al.* 2023; Camras *et al.* 2012; Camras *et al.* 2014; Gao *et al.* 2020; Huang *et al.* 2015; Johnson *et al.* 2015; Lee *et al.* 2021; Li *et al.* 2019; Li *et al.* 2013; Overby *et al.* 2014; Vahabikashi *et al.* 2019; Vargas-Pinto *et al.* 2013; Wang *et al.* 2018; Wang *et al.* 2017b; Yuan *et al.* 2011; Yuan *et al.* 2016; Zeng *et al.* 2010). Details regarding the merits or challenges of each individual method are out of the scope of this chapter, and we refer you to consult other reviews on this subject matter (Acott *et al.* 2021; Wang *et al.* 2017a). Nevertheless, our group has extensively reported the use of atomic force microscopy in characterizing TM cell/ECM/and tissue biomechanical properties (Chang *et al.* 2014; Dhamodaran *et al.* 2020; Last *et al.* 2011; Morgan *et al.* 2014; Morgan *et al.* 2015a; Morgan *et al.* 2015b; Peng *et al.* 2022a; Peng *et al.* 2022b; Raghunathan *et al.* 2017; Raghunathan *et al.* 2015a; Raghunathan *et al.* 2015b; Raghunathan *et al.* 2018; Vranka *et al.* 2018) from human, rabbit, and non-human primate models. In this chapter, we detail the salient steps required for such experiments.

EXPERIMENTAL DESIGN

Operating Principle

Atomic force microscopy is a form of scanning probe microscopy (SPM) whose operating principle (Fig. **1**) is defined by its ability to sense changes on a surface through deflections of a sharp tip (nanoscale) at the end of a cantilever beam (microscale). As the tip contacts the surface, its bends (known as *deflection*, **X**) as a function of force (**F**) exerted by the sample below. The bending of the cantilever is detected by a change in voltage using a laser diode and a split photodetector. Thus, the tip-sample interaction is inherently dependent on how much a cantilever may bend in response to the force (spring constant, **κ**). *i.e.* $\mathbf{F} = \boldsymbol{\kappa} \cdot \mathbf{X}$. The resulting output is known as a Force-Distance or Force-Displacement (F-D) curve.

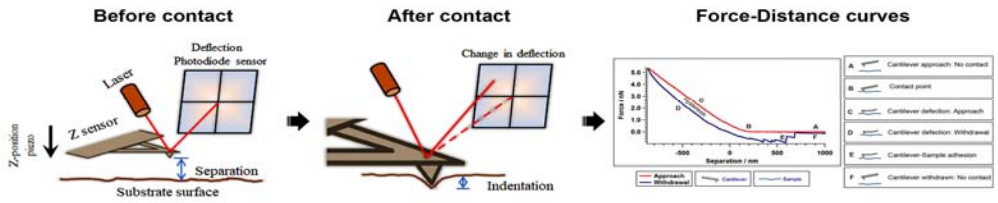

Fig. (1). Principle of operation of an atomic force microscope.

CHAPTER 19

Derivation of Primary Human Trabecular Meshwork Cell-Derived Matrices

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Abstract: Ocular hypertension (OHT) partly results from increased resistance to aqueous humor (AQH) outflow from the anterior chamber of the eye as a result of changes in the extracellular matrix (ECM) of trabecular meshwork (TM). Even though *in vivo* animal models of OHT often rely upon singular gene knockout systems, these do not reveal the complexities in matrix changes (structure and composition) in the disease. Also, i*n vitro* systems mimic the native topography, mechanics, or biochemistry of native ECM exist but often fail to mimic the multifaceted environment around the cells. There is, thus, a paucity of physiologically relevant assays/models to perform non-clinical mechanism-based research. Cell-derived matrices (CDMs) represent a 3D microenvironment derived from a cell type of interest that partially simulates the matrix similar to conditions *in vivo*. To mimic homeostasis under normal conditions or pathological states, the CDM content can be altered by the addition of various classes of compounds. A protocol for generating CDMs from isolated and cultured primary TM cells is described in this chapter.

Keywords: Decellularization, Extracellular matrix, Glaucoma, Matrices, Primary cells, Trabecular meshwork.

INTRODUCTION

Primary open-angle glaucoma (POAG) is a common multifactorial age-associated ocular disease worldwide, which causes irreversible vision loss if left untreated. Elevated intraocular pressure (IOP) is intimately linked to the etiology and progression of POAG. However, IOP-lowering treatments can partially address this risk factor. The trabecular meshwork (TM) is the primary route of egress for aqueous humor (AQH) from the anterior chamber of the eye (Stamer and Acott, 2012). However, abnormally accumulated extracellular matrix (ECM) in and around the TM obstructs the AQH drainage and is the main contributor to the de-

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velopment of ocular hypertension (OHT) (Vranka *et al*., 2015; Acott and Kelley, 2008). Existing experimental systems of OHT have often focused on studying the role of individual components of ECM *via* genetic manipulations or exogenous treatments (*e.g*., the addition of tissue growth factor-beta (TGFβ) or corticosteroids such as dexamethasone). However, the ECM is a mixture of proteins, lipids, biochemical ligands (Dhamodaran *et al*., 2022), and other structural elements that are linked and interact in a complex manner. Thus, in addition to contributing towards structure and physical resistance to AQH flow, ECM regulates cell fate and cellular identity (Humphries *et al*., 2018; Keung *et al*., 2010; Sart *et al*., 2020; Schwartz, 2010; Sima, 2017; Sun *et al*., 2012; Trappmann *et al*., 2012; Tschumperlin *et al*., 2013; Urbanczyk *et al*., 2020; Vining and Mooney, 2017; Vogel and Sheetz, 2009; Wen *et al*., 2014; Wolfenson *et al*., 2019). Consequently, no single ECM component can sufficiently cause a diminution of AQH drainage. Instead, complex interactions among matricellular proteins, growth factors, glycoproteins, and fibrillary and non-fibrillary structural proteins most likely influence the rate of ECM aggregation and digestion and thereby increase the overall stiffness of the TM and the surrounding environment. These events and factors are thus responsible for the raised resistance to AQH efflux from the anterior chamber.

CDMs are composed of a mixture of ECM biomolecules that can potentially recapitulate highly complex extracellular milieu encountered *in vivo* at the level of the TM and offer means to elucidate the relationship between the ECM turnover and cellular functions (Cukierman *et al*., 2001). Described below is a reproducible method to generate primary human TM CDMs that can be utilized to study cellular mechanics and responses to exogenous growth factors and dissect molecular signaling pathways (Raghunathan *et al*., 2015a,b; 2018; Yemanyi *et al*., 2019, 2020a-d). It must be mentioned here that the creation and harvesting of CDMs is a labor-intensive and tedious process that requires patience in order to obtain a cell-free product. Even though ECMs derived from different tissue sources tend to contain a similar core of constituents, each ECM does possess a unique ‘matrisomal’ signature (Naba *et al*., 2012, 2016; Taha and Naba, 2019; Bingham *et al*., 2020; Nerger *et al*., 2022). Consequently, it is imperative that specific procedures and conditions for cell isolation, propagation, and decellularization are delineated to reproducibly and successfully generate the desired CDMs from any particular cell type.

EXPERIMENTAL PROTOCOLS

Reagents and Supplies Required

- Since the current topic is matrices derived from human TM (hTM) cells, the latter cells must first be isolated and cultured from post-mortem human donor eye TM tissue as previously described (Keller *et al*., 2018). Various reagents are needed for this and future purposes and are listed below, including the vendors and their locations.
- Tissue culture-treated dishes, 60 mm diameter (Corning Life Sciences, Corning, NY)
- Fine-tipped sterile plastic transfer pipettes (VWR International, Radnor, PA)
- Round glass coverslips, 12 mm diameter (Ted Pella, Inc., Redding, CA)
- Dulbecco's modified Eagle Medium/Nutrient mixture F-12 (DME/F-12, 1:1); (GE Healthcare Life Sciences, Logan, UT).
- Fetal bovine serum (FBS) (Atlanta Biologicals, Flowery, GA).
- Penicillin/streptomycin/amphotericin-B (Life Technologies, Carlsbad, CA, USA).
- Phosphate buffered saline (PBS, without calcium and magnesium) (GE Healthcare Life Sciences, Logan, UT).
- Versene (EDTA), 0.02%; 0.2 g/L ethylenediaminetetraacetic acid (0.53 mM) in DPBS, without calcium and magnesium (BioWhittaker® Reagents, Lonza, Walkersville, MD)
- 0.05% Trypsin-EDTA (ThermoFisher Scientific, Waltham, MA).
- Ammonium hydroxide (EMD Millipore Corporation, Darmstadt, Germany).
- 3-aminopropyl trimethoxysilane (281778-100ML, Sigma-Aldrich, St. Louis, MO) or 3-aminopropyl triethoxysilane (APTES; Sigma-Aldrich, St. Louis, MO).
- Triton X-100 (VWR International, Solon, OH).
- DNase I (Spectrum Chemical, Gardena, CA).

Reagent Setup

- *Complete growth medium:* In order to culture and propagate primary hTM cells, prepare the growth medium by supplementing DME/F-12 with 2.5mM L-glutamine, 10% FBS, and 1% penicillin/streptomycin/amphotericin-B. Then, refrigerate this culture medium.
- *Decellularization / Extraction buffer:* Prepare the decellularization buffer under aseptic conditions as follows: to 498.5 mL of deionized sterile water, add 1.25 mL of ammonium hydroxide and 250 µL of Triton X-100 detergent and then keep it at the bench.
- *DNase I solution:* Now prepare DNase-I to 50 U/mL. This will be utilized to eliminate DNA from decellularized cell-derived matrices.

CHAPTER 20

Protocol for Studying TM Cell Function in a 3-D Matrix

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Abstract: This chapter describes the protocols to bioengineer a 3D human trabecular meshwork tissue model using Glauconix Biosciences' patented 3D-HTM™ system. These protocols combine species-relevant tissue with nanoengineering techniques in order to produce a 3D matrix to mimic the conventional outflow pathway, which is responsible for the majority of the aqueous humor drainage and intraocular pressure regulation in the eye. Currently, there is a lack of translation between glaucoma in the human eye and animal models, which fails to provide a sufficient platform for compound screening and intraocular pressure (IOP) assessment. To address this unmet need, a 3D-HTM™, which can be co-cultured with human Schlemm's canal cells and induced to a glaucomatous-like state, was developed to provide a more applicable model for the evaluation of IOP-lowering therapeutics. The included protocols address the engineering of this 3D tissue model, as well as the examination of key biological markers of diseased tissue using traditional biological techniques with a few modifications. Additionally, a methodology for indirectly measuring IOP within the bioengineered 3D tissue is described. Overall, these protocols provide an *in vitro* alternative for studying glaucoma at the anterior segment, which can propel the development of glaucoma therapeutics.

Keywords: 3D tissue, Conventional outflow, Eye, Fibrosis, Glaucoma, Intraocular pressure, Ocular hypertension, Schlemm's canal cells, Trabecular meshwork.

INTRODUCTION

The study of the anterior chamber of the eye, as it relates to the understanding of glaucoma and fibrosis, prompted the development of relevant 3D human glaucomatous tissue models for physiological and species-relevant research. The human trabecular meshwork (HTM) plays a critical role in maintaining intraocular pressure (IOP) and contributes to the maintenance of vision (Lütjen-

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Drecoll, 1999; Zhou, Li and Yue, 1999; Llobet, Gasull and Gual, 2003). It is a sieve-like filtering structure that regulates the flow of aqueous humor to maintain IOP (Llobet, Gasull and Gual, 2003; Abu-Hassan, Acott and Kelley, 2014). The HTM is comprised of distinctive regions: uveal meshwork, corneoscleral, juxtacanalicular, inner wall of Schlemm's canal, and Schlemm's canal (Lütjen-Drecoll, 1999; Zhou, Li and Yue, 1999; Llobet, Gasull and Gual, 2003). The region believed to provide the most resistance to aqueous humor and hence linked to the regulation of intraocular pressure is the Juxtanacanlicular region and the inner wall of the Schlemm's canal. This area is comprised of human trabecular meshwork cells sitting on a basement membrane and interacting with Schlemm canal cells (Llobet, Gasull and Gual, 2003; Abu-Hassan, Acott and Kelley, 2014). The critical role of each of these components of the trabecular meshwork has been discussed in chapter 2 of this book. Bioengineering this structure enables studying key HTM and Schlemm's canal markers that may lead to novel targets for the treatment of ocular hypertension. In this chapter, we detail protocols to enable the monoculture of HTM cells on a basement membrane-like structure, coculture with Schlemm's canal cells for a more physiologically relevant representation of the anterior region, and creation of a disease-like state to mimic fibrosis and glaucoma. Finally, we discuss methods to evaluate IOP *in vitro.*

EXPERIMENTAL PROTOCOLS

Preparation of the Basement Membrane-Like Scaffold for the Culture of HTM and Schlemm Canal Cells

- Porous SU8 scaffolds are fabricated using established photolithography techniques, as seen in Fig. (**1**) below.
- Aluminum tape rings are made using a Cricut machine to the desired diameter of interest. Scaffolds are affixed to the rings in the biosafety cabinet by gently picking up the scaffold with tweezers and placing it onto the sticky side of an aluminum ring (see Fig. **2** below).
- The mounted scaffolds are sterilized by using 70% ethanol and allowed to dry for at least 20 minutes, followed by UV exposure for 1 hour.
- The sterile scaffolds are gelatin-coated by allowing them to be in direct contact with 1% gelatin and air-dried for 20 minutes overnight in the biosafety cabinet. These scaffolds can then be placed in 24 well plates, covered with Parafilm™, and stored at 4 °C until ready for cell culture.

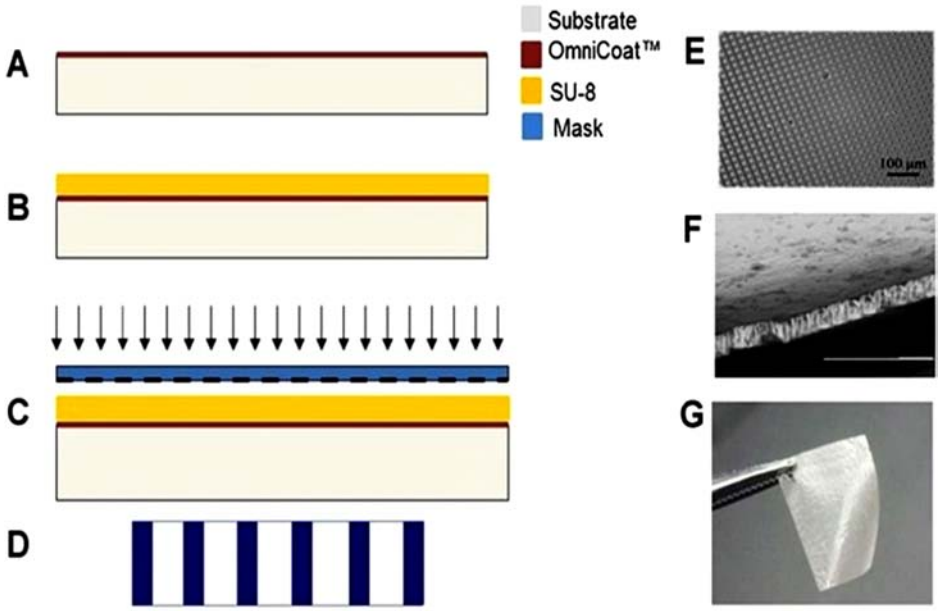

Fig. (1). Schematics of the fabrication of the micropatterned, porous SU-8 scaffold using photolithograpy. A: OmnicoatTM treatment on previously cleaned silica wafer for the release layer. B: SU-8 2010 coating. C: Exposure and post-exposure bake. D: Development. Representative scanning electron micrographs of top view (E) and cross-section (F), and optical image (G) of the freestanding scaffold. (E and F) Scale bar = 100 µm. (Torrejon *et al*., 2013).

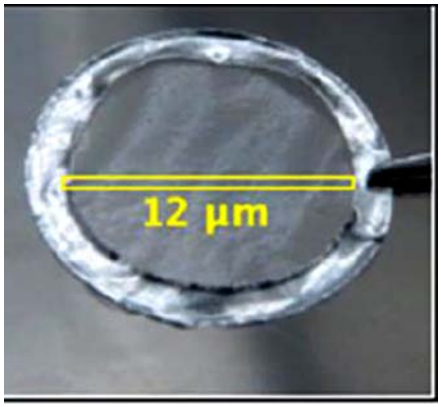

Fig. (2). Image of Glauconix Biosciences' patented SU-8 scaffold affixed to an aluminum ring. Diameter =12 µm.

Culture of Primary HTM Cells on Gelatin-coated SU8 Porous Scaffolds

- Primary HTM cells, isolated from the juxtacanalicular and corneoscleral regions of healthy human eyes, are plated in a T75 flask coated with 1% gelatin and supplemented with a 10% heat-inactivated FBS-IMEM medium. The medium is replaced every other day until the cell reaches confluency.

CHAPTER 21

Protocols for Studying Neurotransmitter Release from Isolated Ocular Tissues

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Abstract: Neurotransmitters mediate signals from nerve terminals to the postsynaptic receptors within central and peripheral nervous systems. The release of the transmitters depends on the action potential within the cognate nerves. Herein, protocols are described for measuring the release of neurotransmitters (such as norepinephrine) from isolated anterior uveal tissues (such as the iris/ciliary bodies), which influence the production and secretion of aqueous humor (AQH). The production and drainage of AQH and the ultimate volume within the anterior segment of the eye determine the intraocular pressure (IOP). Isolated neural retinal tissues can also be used to study neurotransmitter release under conditions where there is a need to assess the ability of compounds to offer neuroprotection in the eye. For instance, compounds that can prevent the release of glutamate from the retinal slices or whole tissue may prove to be neuroprotective by preventing neurodegeneration. The use of radiolabeled neurotransmitters to mimic the physiological actions of their endogenous counterparts and the release of the tritiated transmitter, for example, can be triggered using an electrical field stimulus, a potassium-depolarizing pulse, and hypoxia and glucose-deprivation, for instance, can emulate ischemic conditions *in vivo*. Protocols for measuring the release of radiolabeled norepinephrine and serotonin from iris/ciliary bodies of various species will be described in this chapter. Additionally, protocols for measuring radiolabeled D-aspartate release from the neural retina of these species will also be discussed.

Keywords: Ciliary body, Iris-ciliary body, Release mechanism, Retina, Transmitter.

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INTRODUCTION

Communication between nerves and effector cells is often mediated *via* neurotransmitters that are released by depolarization of the nerve terminal evoked by the arrival of action potentials in the peripheral and central nervous system (CNS). This process depends on the membrane potential at the nerve terminal and on the availability of stored neurotransmitters. Transmitter release parameters can be studied both *in vitro* and *in vivo,* and the effect of drugs and exogenous agents on such processes can be measured and quantified. A major focus of pharmacological studies using drugs that interfere with the release of neurotransmitters is their ability to alter the activity of presynaptic and/or postsynaptic receptors, thereby influencing the final biological response, whether it be hormone release, tissue contraction, or induction of sleep.

Presynaptic receptors act as a homeostatic control switch where they can either inhibit or enhance the release process. The depolarizing stimulus, whether electrical or chemical, may be employed continuously or intermittently, and the process of neurotransmitter release is measured in a super-fusion or a static system. The measurement of neurotransmitter release requires the rapid dissection of tissues from appropriate organs and immediate transfer to oxygenated buffer solutions. For the static system, it is important to gently agitate the incubation medium to enable adequate oxygenation and diffusion of endogenous materials/chemicals into the bathing medium. With the super-fusion system, flow rates of 0.25 to 2 ml/min are used to allow an adequately oxygenated and stable environment for the tissues. In order to enable adequate equilibration and stabilization of tissues under study in either of the static or super-fusion systems, it is highly recommended that tissues undergo a 20-60 minute preincubation at room temperature. The tissues should then be gently washed by a series of gentle centrifugation/filtration steps to remove endogenous transmitter substances before the actual experiments begin. In a static system, the measurement of neurotransmitter release is performed in test tubes or beakers. For the super-fusion system, a custom-made chamber to place the washed tissues is utilized with appropriate connections to a peristaltic pump using flexible tubing. Studies on the *in vitro* release of neurotransmitters from static and super-fusion systems have utilized the radiolabeled form of a transmitter of interest to enable their uptake and storage *via* selective and high-affinity carriers within the tissue nerve terminals or cells. Quantification of release of endogenous pools of neurotransmitters can be determined using selective and sensitive analytical tools such as high-pressure liquid chromatography (HPLC) or liquid chromatography with tandem mass spectrometry (LC-MS-MS).

OVERALL GENERAL EXPERIMENTAL PROCEDURES

Studies on neurotransmitter release from isolated ocular tissues involve the use of iris-ciliary bodies and/or the neural retina. All experiments using animals should be in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology. Approval of protocol for the use of human tissue should be obtained from the Institutional Review Board prior to the acquisition and use of postmortem human eyes.

Experiments Using Electrical Stimulus to Induce the Release of Radiolabeled Neurotransmitters from Isolated Iris/Ciliary Bodies

Tissue Preparation

Eyeballs from freshly sacrificed cows, pigs, or rabbits should be kept on ice until ready for dissection. Human eyeballs should be obtained within 6-8 hours post-mortem and can be stored in sealed vials containing oxygenated Krebs buffer solution at 2-4°C for up to 12 hours. While the whole iris/ciliary bodies from rabbit, pig, and human eyes can be mounted in specially designed perfusion chambers for neurotransmitter release studies, iris/ciliary bodies from cows can be divided into 4 quadrants for ease of fit into the chambers. [^{3}H]- 5-hydroxytryptamine ([^{3}H]-5HT, 1.6 μM) and [^{3}H]-norepinephrine ([^{3}H]NE, 2.5 μCi/ml) can be used for neurotransmitter uptake and release studies using the iris/ciliary bodies.

Protocol for Measuring [^{3}H]NE release from Anterior Uveal Tissues

The procedures used in studies on [^{3}H]NE secretion from the iris/ciliary bodies have been previously described by several investigators (Ohia and Jumblatt, 1990; Ohia and Jumblatt, 1991; Salvi *et al*., 2016). Briefly, freshly isolated bovine, porcine, rabbit, or human iris-ciliary bodies (ICB) are incubated with 4 ml of well-oxygenated Krebs buffer solution containing 0.1-1 μM [^{3}H]-NE at 37^{o}C for 1 hour. The Krebs buffer solution is composed of (mM) NaCl 118, KCl 4.8, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 2.0, and dextrose 10 (pH 7.4). Ascorbic acid (56.7mM) is added to Kreb's buffer solution, and in some experiments, flurbiprofen (FBF 3 μM), a cyclooxygenase (COX) inhibitor, is added to the buffer to reduce the contribution of endogenously produced prostaglandins to the study. After this incubation, tissues are gently rinsed twice with fresh Krebs buffer for 5 minutes each, mounted between nylon mesh cloth, and placed in thermostatically controlled superfusion chambers (Warner Instrument Corp., CT). Tissues are then super-fused with oxygenated Krebs buffer solution at a rate of 2 ml/min, with effluent buffer being collected at 4-minute intervals.

CHAPTER 22

Protocols for Studying Contraction/Relaxation of Ocular Tissues *In Vitro*

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Abstract: Tissues isolated from organs have been extensively used in pharmacology or toxicology to study the effects of chemicals and drugs. A major advantage of using isolated tissues (instead of studying responses *in vivo*) is the exclusion of connective tissues, hormonal influences, or homeostatic mechanisms that may interfere with responses expected of the tissue under experimentation. The use of isolated tissues from the eye for physiological/pharmacological studies can yield significant amounts of information that is pertinent to the identification of new pathways or processes. Indeed, the ability to use various isolated ocular smooth muscles (iris, ciliary, vascular), skeletal muscle (rectus), and the TM to study contractions/relaxations to a wide range of drugs and chemicals provides a unique opportunity to identify potential new drugs for the treatment of diseases of the anterior segment such as glaucoma.

Keywords: Connective tissues, Glaucoma, Homeostatic mechanisms, Hormonal influences, Ocular smooth muscles, Ocular tissues, Skeletal muscles.

INTRODUCTION

Tissues isolated from organs have been extensively used in pharmacology or toxicology to study the effects of chemicals and drugs. A major advantage of using isolated tissues (instead of studying responses *in vivo*) is the exclusion of connective tissues, hormonal influences, or homeostatic mechanisms that may interfere with responses expected of the tissue under experimentation. In the eye, muscles (smooth and striated) have been isolated for use in studying the responses to chemicals or drugs. Strips of the trabecular meshwork have also been used for the measurement of contractile responses initiated by the activation of pharmacological receptors. Both contraction and relaxation of ocular tissues can be assessed using transducers that can detect changes in isometric tension developed or changes in the length of tissues in response to force (isotonic ten-

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sion). Isolated ocular tissues should be placed immediately in a physiological salt solution with a composition that closely mimics the internal environment. For instance, Krebs buffer solution is used for most isolated smooth muscle preparations, while Ringer's solution is used for isolated trabecular meshwork strips. Typically, isolated tissues placed in physiological salt solutions should be maintained at a temperature that closely mimics the internal one (depending on the tissue) and gassed with appropriate gas (such as oxygen, carbon dioxide, or air). When used under the appropriate conditions, isolated ocular tissues can remain viable for more than six hours.

GENERAL EXPERIMENTAL PROTOCOLS

Experiments using isolated ocular tissues such as iris/ciliary muscles, trabecular meshwork, and blood vessels from rabbits, pigs, cows, and humans can be performed using specialized tissue/organ baths. After procurement from the slaughterhouse or Eye Banks, the eyeballs should be transported to the laboratory on ice. Careful dissection of tissues should lead to the isolation of the iris/ciliary muscles, trabecular meshwork, and blood vessels from an eyeball. All experiments using animals should be in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology. Approval of protocol for the use of human tissue should be obtained from the Institutional Review Board prior to the acquisition of human eyes.

MEASUREMENT OF CONTRACTION/RELAXATION OF ISOLATED OCULAR SMOOTH MUSCLES

Tissue Preparation

Eyeballs from freshly sacrificed cows, pigs, or rabbits are transported to the laboratory on ice. When used, human eyeballs should be procured within six hours post-mortem and can be stored in sealed vials containing oxygenated Krebs buffer solution at 2°C for up to 12 hours. Approval of protocol for the use of human tissue should be obtained from the Institutional Review Board prior to the acquisition of human eyes. The iris smooth muscle/ciliary muscle can be dissected out of eyeballs from different species; whole irises/ciliary muscles from pigs, humans, or rabbits can be used, while each whole irise from cows can be cut into half for experiments. All dissected tissues should be placed in Krebs buffer solution in preparation for the experiment.

Protocol for Measuring Contraction/Relaxation of Iris/Ciliary Smooth Muscles

The methodology used for studies on the contraction/relaxation of isolated iris of pigs and ciliary muscle of cows are described by us and other investigators (Ohia *et al*., 2010; Romano and Lograno, 2013; Ohia *et al*., 2018.). For pigs, whole irises are isolated, placed in a Krebs buffer solution, and prepared for contraction/relaxation studies. For cows, ciliary muscle from each eyeball is quickly dissected under a binocular microscope, and 4-5 mm length is placed in Krebs buffer solution, which is then prepared for contraction/relaxation studies. After the setup of tissues in a thermostatically maintained (37^{o}C) organ bath containing oxygenated (95% O_2: 5% CO_2) Krebs solution (pH 7.4). The Krebs solution should have the following composition (mM): potassium chloride, 4.8; sodium chloride, 118; calcium chloride, 2.3; potassium dihydrogen phosphate, 1.2; sodium bicarbonate, 25; magnesium sulfate, 2.0; and dextrose, 10. The muscle preparation in the organ bath is then connected to a force-displacement transducer (Ugo Basile, Stoelting Co., Wood Dale, IL 60191, USA) under resting tensions of 150 mg (for porcine tissues) and 300 mg (for bovine tissues). The tissues are then allowed to equilibrate in the oxygenated Kreb buffer solution in the organ bath for 60 minutes (with bath fluid replaced every 15 minutes). Changes in isometric tension of the tissues are captured using the computer software Digital Recorder DataCapsule-Evo, Stoelting Co., Wood Dale, IL 60191, USA). Before the addition of any drug, all muscle strips should be given a submaximal concentration of the muscarinic receptor agonist, carbachol, to check for the functionality of the tissues. After equilibration, contraction of the muscles can be elicited using different concentrations of muscarinic receptor agonists such as carbachol. To study the relaxant response of tissues, tone can be induced with a contractile agent that can maintain sustained tone (*e.g*., carbachol), enabling the study of responses of different concentrations of an inhibitory agent (*e.g*., β-adrenoceptor agonist such as isoproterenol) on the induced tone. For pharmacological studies, both contractile concentration-response and relaxant concentration-response curves can be constructed in this system. After analysis of the data obtained under control and test conditions, relevant pharmacological information (such as the EC_{50} or IC_{50} values) can be obtained.

MEASUREMENT OF CONTRACTION/RELAXATION OF ISOLATED OCULAR VASCULAR SMOOTH MUSCLES

Tissue Preparation

Posterior ciliary arteries can be dissected from cows, rabbits, and human eyeballs (Kulkarni-Chitnis *et al*., 2015; Chuman *et al*., 2017.). For all species, the entire

CHAPTER 23

Protocol for Studying Aqueous Outflow Facility from Anterior Eye Segments *In Vitro* as an Organ Culture Procedure

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Abstract: Open-angle glaucoma is a neurodegenerative eye disease that is associated with elevated intraocular pressure (IOP) and affects millions of people worldwide. Since the raised IOP is the only risk factor that can be treated, much effort is being expended in finding novel ways to promote the efflux of aqueous humor (AQH) from the anterior chamber (ANC) of the eye. This is important for lowering and controlling IOP in order to prevent the loss of retinal ganglion cells and their axons, thereby slowing down vision loss. Even though several animal models have been developed to study the efficacy of new compounds to reduce ocular hypertension (OHT), such studies are labor-intensive and expensive. An in vitro model to permit similar kinds of investigations has been created using ANC segments of animal and human postmortem eyes. This system capitulates the *in vivo* situation and is easier to use and less expensive than the animal models. An organ culture model system and techniques will be described in this chapter.

Keywords: Aqueous humor, Glaucoma, Intraocular pressure, Ocular hypertension, Perfused eye.

INTRODUCTION

Excessive accumulation of aqueous humor (AQH) in the anterior chamber of the eye is often associated with different forms of glaucoma, a constellation of neurodegenerative diseases with varied etiologies. The retention of AQH in the ANC results from pathological blockage of the main drainage system for the fluid, the trabecular meshwork (TM), by excessively deposited collagen, fibronectin, and myocilin proteins (Polansky *et al*., 2000; Weinreb *et al*., 2014). Over time, the

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pressure in the ANC rises and is transmitted throughout the eye, leading to disturbances in the structure and function of retinal ganglion cells and their axons (which form the optic nerve), thereby resulting in visual impairment (Weinreb *et al*., 2014; Bucolo *et al*., 2018; Sharif, 2021). If left untreated, this ocular hypertension (OHT) can lead to irreversible loss of vision.

The mainstay treatment for OHT and such glaucomatous optic neuropathy is the lowering and controlling of the intraocular pressure (IOP) by pharmaceutical (*e.g*. topical instillation FP- and EP2-receptor agonist drugs or other ocular hypotensive agents) implantation of microshunts to remove the excess AQH and surgical means (Bucolo *et al*., 2018; Sharif, 2021; Sharif *et al*., 2023). Due to patient incompliance and side effects of existing treatments, new drugs to tackle OHT and glaucoma are needed. Screening of potential drug candidates for ocular hypotensive activity relies on animal models of OHT, which is an expensive and labor-intensive endeavor. To reduce animal use, increase the throughput, and lower the cost of testing compounds, the use of ANC segments of animal and human eyes postmortem can be a viable alternative (Johnson *et al*., 1990; Clark *et al*., 1995; Mao *et al*., 2011). Such procedures can also provide mechanistic information about the test agents in these organ cultures of ANC segments (Webb *et al*., 2006; Mao *et al*., 2011).

Perfused Organ Culture Anterior Segments (POCAS) from human post-mortem or from a number of different animal species have been a standard model for examining the aqueous outflow pathway for over 30 years (Johnson *et al*., 1990; Clark *et al*., 1995). This ex-vivo model preserves the architecture of the aqueous outflow pathway and enables studies to be performed in a near physiologic state. Typically, POCAS are perfused in pairs from the same individual with one eye serving as control while the other as experimental control. This somewhat reduces the variability in the outflow facility measurements. For all perfused POCAS studies (irrespective of the species used), it is important to perform a histological examination of the outflow tissues after perfusion to ensure no gross disruption of the outflow pathways, which can dramatically affect the results occurred during the experimentation (Johnson *et al*., 1990; Mao *et al*., 2011). Histological examination should also be used to verify that the TM cellularity is within acceptable bounds, as loss of TM cells can lead to preparations that are not responsive to pharmacologic agents. It is also important to point out that perfused POCAS from many animal species (including those from primates) exhibit the "washout effect". This is an increase in outflow facility over time during the initial period of perfusion, which occurs in perfusion models and has been attributed to the removal of extracellular cells or ECM components in outflow pathway tissues. Whether washout is time- or volume-dependent is still not clear (Mao *et al*., 2011). Although different animal species show varying amounts of

this effect, the washout effect is absent in human and probably mouse eyes. The use of a POCAS-based organ culture system is described below.

Tissue Acquisition and Preparation

- Obtain enucleated animal or human eyeballs as fresh as possible from the appropriate source with due consent and related administrative paperwork and keep them on ice.
- Bisect the eye equatorially and carefully remove the vitreous, lens, and iris.
- Mount the anterior segment in a sterile Plexiglass culture dish and seal with a Plexiglass O-ring (Fig. **1**).
- Perfuse the eye segment with chemically defined media (Dulbecos's modified Eagle's medium; Sigma Chemical Co., St. Louis, MO) containing L-glutamine, penicillin, streptomycin, and gentamicin (Gibco/BRL, Grand Island, NY) at a fixed flow rate of 2 /xl/minute using a Harvard perfusion pump (Harvard Instruments, Cambridge, MA).
- Connect a pressure transducer (Stemtech, Houston, TX) connected to the second cannula and monitor the IOP within each eye. In this perfusion system, the perfusate exits the eye through the conventional aqueous humor outflow system. The TM cells of the outflow pathway remain viable for up to 4 weeks in the culture.
- After stabilization for 2-3 days, the test compound can be added to the culture media of one eye while the contralateral eye serves as the control, receiving an equivalent volume of the vehicle during the perfusion.
- Monitor and record the IOPs over several minutes/hours depending on when the peak and plateau phase is achieved.
- If mechanistic studies are undertaken, such as determining the role of matrix metalloproteinases (MMPs) in the molecular mechanism of action of a drug (Webb *et al*., 2006), then inhibitors of MMPs can be added to the perfusion medium 20-30 minutes before the test compound is introduced. Appropriate controls for experimental arms would need to be designed and implemented. The MMPs can also be co-added with the drug and maintained in a perfused combination throughout the experiments. Here, samples of the perfusate are taken at various time points and later analyzed for Pro-MMPs before, during, and after the perfusion is commenced.
- Likewise, if the effect of corticosteroids on the IOP and outflow facility needs to be determined, then the dexamethasone (0.1-1 uM) would need to be added to the perfusion medium and the experiment continued (with appropriate controls) for 2 hours (Clark *et al*., 1995; Mao *et al*., 2011). The structural changes in the TM and adjoining tissues can then be morphologically determined, as outlined below.

CHAPTER 24

Human TM Cells as Models for Pseudo-Exfoliation Glaucoma (PEXG)

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Abstract: Exfoliation syndrome and exfoliation glaucoma (XFG; PEXG) remain an enigma with regard to their pathogenesis and cause of glaucoma. While several studies have characterized their clinical characteristics and different phenotypic variations, the mechanisms of formation for complex protein aggregates within ocular tissues like the trabecular meshwork (TM) in this disease entity are largely unknown. The risk factors for the onset of glaucoma in XFS remain a mystery, with only selected eyes developing glaucoma or raised intraocular pressure, IOP. Several molecular markers are known to be upregulated in XFS and XFG eyes, including oxidative stress markers, cytokines, and transforming growth factor (TGF-β1). Several studies have elucidated the differences between eyes with exfoliation and primary glaucoma. Yet, the molecular events that mark the onset of XFS or the development of glaucoma are under-investigated. This is largely because of the inability to sample ocular tissue like the TM or iris in early disease stages. The variable viability of cells from tissues obtained during surgery is a concern precluding easy understanding of the events involved specifically in this disease. In vitro human TM cell line models mimicking this disease allow us to study the temporal events that may mimic the disease's onset and progression over time. We herein present the protocols for establishing such an in vitro model for the study of exfoliation syndrome or XFG, which may be valuable for evaluating various molecular events responsible for the disease pathogenesis.

Keywords: Cell line model, Exfoliation syndrome, Exfoliation glaucoma, Human trabecular meshwork, Transforming growth factor.

INTRODUCTION

The trabecular meshwork (TM) is a tissue located in the eye that helps regulate the outflow of fluid from the eye and maintain normal intraocular pressure (IOP) (Fig. **1**). This is the primary site of injury in glaucoma caused by exfoliation syn-

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drome (Rao *et al*., 2017; Rao *et al*., 2014; Schlotzer-Schrehardt *et al*., 1995; Sahay *et al*., 2021; Garweg *et al*., 2017). In glaucoma, elevated IOP can damage the optic nerve, leading to vision loss. As a result, the TM has been studied as a cellular model for understanding the pathophysiology of glaucoma and developing treatments for the disease (Schlotzer-Schrehardt *et al*., 1995; Sahay *et al*., 2021; Chakraborty *et al*., 2021; Lee *et al*., 2008; Evangelho *et al*., 2019; Sahay *et al*., 2017). The TM is composed of various types of cells, including extracellular matrix (ECM) proteins and various types of cells, such as fibroblasts, myofibroblasts, and inflammatory cells (Schlotzer-Schrehardt *et al*., 1995; Chakraborty *et al*., 2021). Studies using in vitro and ex vivo models of the TM have provided valuable insights into the cellular and molecular changes that occur in glaucoma and how they may contribute to the progression of the disease (Chakraborty *et al*., 2021; Evangelho *et al*., 2019). These models have also been used to test new therapies for glaucoma, such as drugs that target specific signaling pathways or ECM proteins involved in the regulation of IOP.

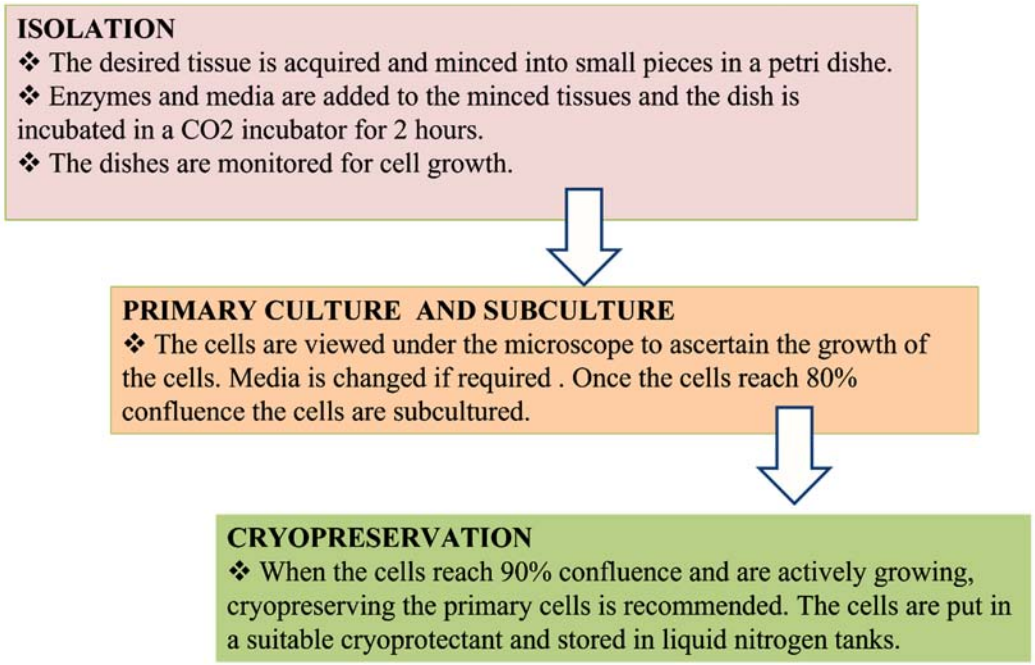

Fig. (1). Anatomy of the eye showing the trabecular meshwork and its relation to adjacent angle structures.

Exfoliation syndrome (XFS) or exfoliation glaucoma (XFG) represents a unique entity among all glaucoma types characterized by the presence of dandruff-like protein complex aggregates within the eye (Schlotzer-Schrehardt *et al*., 1995; Sahay *et al*., 2021; Chakraborty *et al*., 2021; Lee *et al*., 2008; Evangelho *et al*.,

2019; Sahay *et al*., 2017). These aggregates cause gradual obstruction of the TM and outflow channels that cause tissue dysfunction and TM cell death over time (Rao *et al*., 2017; Rao *et al*., 2014). This causes raised intraocular pressure and irreversible optic nerve damage, leading to blindness. This disease entity has evaded a clear explanation of its pathogenesis on how the aggregates form or what marks the onset of glaucoma. While the TM is the primary site of injury, sampling the TM in early disease stages is unethical, which makes the study of the molecular events causing tissue death impossible. This is precisely why in vitro models using HTM cell lines are important to studying specific temporal molecular events. Transforming growth factor (TGF β1; TGF) is known to be upregulated in XFS and XFG (Jampel *et al*., 1990; Zenkel *et al*., 2014; Hao *et al*., 2019; Rao *et al*., 2020; Chakraborty *et al*., 2022; Sahay *et al*., 2022). This is known to regulate key processes involved in TM cell repair and cell function, which get dysregulated by exfoliation deposits and result in TM dysfunction and glaucoma over time (Sahay *et al*., 2017; Chakraborty *et al*., 2022; Sahay *et al*., 2022). An in vitro model of exfoliation syndrome refers to a laboratory-based system for studying this disease outside of the human body (Chakraborty *et al*., 2021; Lee *et al*., 2008; Evangelho *et al*., 2019; Sahay *et al*., 2017). In vitro models are typically created using cell cultures or tissue samples obtained from healthy donors or patients with exfoliation syndrome. These models allow researchers to study the disease in a controlled environment, where various conditions and treatments can be precisely controlled and monitored. Though they may not simulate the actual disease that happens in vitro, they provide valuable insights into events that mark the onset of changes that cause cell dysfunction or cell death. They also give specific therapeutic targets to reverse these changes that may be used for preventing the onset of glaucoma or disease progression. The in vitro model of exfoliation syndrome has been used to study the cellular and molecular changes that occur in the disease and to test new treatments for exfoliation syndrome (Chakraborty *et al*., 2021; Lee *et al*., 2008; Sahay *et al*., 2017). For example, researchers may use the model to study the effects of drugs on the expression of specific genes or the accumulation of exfoliation material in the lens capsule. Advantages of using an in vitro model of exfoliation syndrome include the ability to precisely control and monitor various conditions, the availability of large amounts of tissue for analysis, and the ability to study the tissue over an extended period. In conclusion, the in vitro model of exfoliation syndrome is a valuable tool for studying the pathophysiology of the disease and developing new treatments for exfoliation syndrome. We present a simple method of harvesting, creating a primary human trabecular meshwork cell line model for exfoliation with specific treatments, and suggest experiments that may be required to validate the same.

ANIMAL MODELS OF OCULAR HYPERTENSION (OHT)/GLAUCOMA FOR DETERMINATION OF AQUEOUS HUMOR DYNAMICS

CHAPTER 25

Protocol for Inducing IOP-Elevation (OHT) Using Hypertonic Saline Injection into Episcleral Veins of Rats

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Abstract: This chapter provides a description of protocols utilized to develop ocular hypertension (OHT) in rats *via* episcleral/limbal veins occlusion using hypertonic saline injections. A procedure for measuring the elevated intraocular pressure (IOP) using a TonoLab rebound tonometer in these animals is also described. Furthermore, the impact of the raised IOP on retinal ganglion cell (RGC) function in this model of OHT/glaucoma, using pattern electroretinograms (Pattern ERG) as a measure of RGC function, is detailed.

Keywords: Episcleral veins, Glaucoma, Ontraocular pressure, Ocular hypertension, Pattern electroretinogram.

INTRODUCTION

It is now well-established that elevated intraocular pressure (IOP) is one of the major risk factors for developing open-angle, closed-angle, and many secondary forms of glaucoma (Weinreb *et al*., 2014; Sharif, 2017, 2021). In order to decipher the cellular and molecular factors and events that cause ocular hypertension (OHT) due to abnormal accumulation of excess aqueous humor in the anterior chamber of the eye, a number of animal models of OHT/glaucoma have been generated. Some of these are described in other chapters in this book. The current chapter focuses on the classic method to induce elevated IOP, *i.e.*, by injecting hypertonic saline into the episcleral veins of lightly anesthetized rodents, especially in rats (Johnson *et al*., 2005; Morrison, 2007; Pazos *et al*., 2016; Morrison *et al*., 2018). The author's own work in this arena of research where this rat model of OHT was developed in Brown Norway rats is described below and

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detailed in a number of publications (Husain, 2012, 2021; Abdul *et al*., 2013; Zaidi *et al*., 2020).

OHT/Glaucoma Model: Rat Episcleral/Limbal Vein Occlusion Model of IOP Elevation

The rat handling and housing were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic & Vision Research, and the study protocol was approved by the Animal Care and Use Committee at MUSC. Brown Norway rats (~150–225 g body weight) were kept under a standard cycle of 12-hours:12-hours light/dark. The following procedure was used to raise IOP in rats.

Micro Injections Procedure

- First, we prepared a micro-injection assembly to inject hypertonic saline into the episcleral/limbal veins. The micro-injection apparatus consisted of 3 components: (1) a glass microneedle ~2-3 mm long with 50 micrometers in diameter, (2) a PE-50 polyethylene tubing (length can vary as needed), and (3) a 23-gauge needle attached to 2-5 ml syringe with a blunt tip. These components were assembled under a dissecting microscope using a 3-way stopcock. This microneedle assembly was attached with a 2 mL syringe, which was further attached with a 2-5 mL syringe with a 3-way stopcock to provide sufficient fluid pressure at the needle tip, as shown in Fig. (**1**).
- Prior to any intraocular pressure (IOP) elevation, a stable baseline IOP was determined.
- Once a stable IOP was determined, IOP was raised by injecting hypertonic saline injections. Rats were then anesthetized with ketamine (75 mg/kg) and xylazine (8 mg/kg), and their body temperature was maintained at 37ºC using a heating pad. Next, a drop of topical proparacaine (0.5%) was applied to numb the cornea 5 minutes prior to the ocular injections.
- The microneedle was held by a pair of curved forceps and pointed toward the limbus. It was then carefully positioned parallel to the vessel wall and slowly inserted into a circumferential limbal vein close to the cornea.
- About 50 μL of 2.0 M hypertonic saline solution (NaCl) was then injected into the limbal venous system as described earlier (Husain, 2012, 2021; Abdul *et al*., 2013; Zaidi *et al*., 2020). For the saline injection, a gentle force was used to sufficiently blanch the limbal artery. The blanching of the circular limbal vein is an indication of proper infusion of hypertonic saline into the veins. Generally, when a greater force is used, it results in excessive IOP elevation, which is detrimental to the subsequent experimental paradigm.

- A little neomycin (an antibacterial ointment) was applied at the injection site of each animal to prevent infection. The rats were kept on the heating pad, monitored, and allowed to recover from anesthesia.
- To control potential pain, animals were given a recommended dose of buprenorphine after surgery, and we continued the analgesic injections for the next 24 hours at an interval of 6-8 hours.
- Animals were observed for general activity and weighed and their IOP was measured on a regular basis (*i.e.*, 3, 7 days, and weekly afterward).
- The anterior chamber was periodically examined to check for potential inflammation.
- In general, a significant elevation in IOP was seen on day 7 and onwards.
- If no changes in IOP were detected after 2 weeks, a second injection of hypertonic saline was performed in an episcleral vein as described above.
- The optic nerve head and retinal vasculature were also assessed by viewing the fundus with a direct ophthalmoscope through pupils dilated with 1% tropicamide and 2% phenylephrine hydrochloride.
- To measure changes in IOP and retinal ganglion cell (RGC) function, IOP and pattern electroretinograms (Pattern-ERG) were routinely measured at an interval of 1-2 weeks post-surgery.

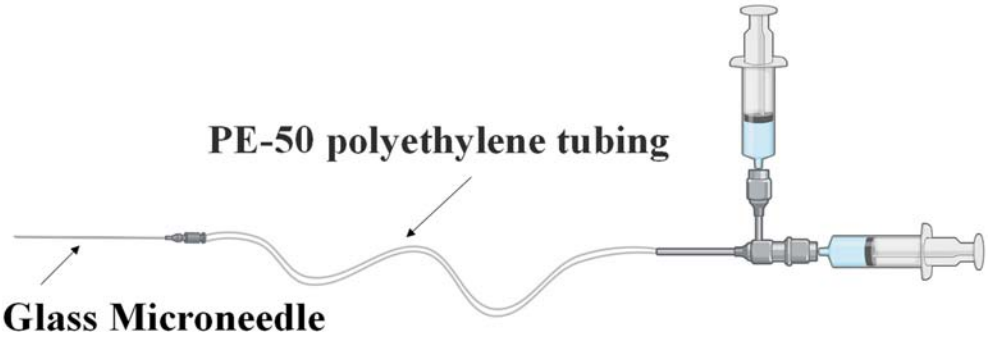

Fig. (1). Schematic showing microinjection assembly.

Measurement of Intraocular Pressure (IOP) in Rats

- IOP was measured in conscious animals in a masked fashion using the TonoLab rebound tonometer (Colonial Medical Supply Co. Inc., Franconia, NH).
- To minimize any discomfort to the animal during tonometry, corneas were lightly anesthetized by the topical application of 0.1% proparacaine (3 μl).
- Baseline IOP readings of both eyes were determined. To ensure the accuracy of the tonometer, the TonoLab read-outs were calibrated against the rat IOPs set by perfusing the rat anterior chamber using saline from a reservoir set at various heights.

CHAPTER 26

Protocol for Inducing Ocular Hypertension in Mice Using Magnetic Microbeads

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Abstract: In many types of glaucoma, a set of chronic neurodegenerative diseases of the retina and optic nerve, elevated intraocular pressure (IOP) is a major culprit. In order to study the disease mechanisms and help discover novel drugs and devices that lower and control IOP, several animal models of ocular hypertension (OHT) have been developed. This chapter focuses on and describes protocols to induce OHT in mice (or rats) using magnetic microbeads (MMBs). Intracameral injection of a suspension of MMBs occludes the trabecular meshwork and prevents or drastically reduces the outflow of aqueous humor (AQH). This gradually raises the IOP. If the IOP is not elevated by 2-fold from baseline and maintained for at least 4 weeks, additional microbeads may need to be injected. Many researchers have successfully created such animal models of OHT and shown them to be useful for drug screening for IOP-lowering efficacy and glaucomatous optic neuropathy studies aimed at finding suitable neuroprotective therapies to preserve vision.

Keywords: Anterior chamber, Eye, Glaucoma, Intraocular pressure, Magnetic microbeads, Ocular hypertension, Optic nerve, Protocol, Retina.

INTRODUCTION

Humans and animals suffer from numerous eye diseases and disorders. Animal models of ocular disease play a central role in defining the factors involved in the pathogenesis of human disease. They also provide a useful model to assess the *in vivo* efficacy of pharmacological agents that may be beneficial in treating the human condition. Accordingly, since glaucoma is a major ocular degenerative disease of concern and varying etiologies, and elevated intraocular pressure (IOP) is a leading cause of many forms of glaucoma (Weinreb *et al.*, 2014; Sharif, 2018, 2021), several animal models have been developed where ocular hypertension

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(OHT) has been induced acutely or on a more chronic basis (Urcola *et al.*, 2006; Morrison *et al.*, 2011; Bouhenni *et al.*, 2012). Many such animal models have been described, including those using latex microbeads to raise IOP (Weber and Zelenak, 2001; Cone *et al.*, 2010; Sappington *et al.*, 2010; Samsel *et al.*, 2011) and more recently utilizing magnetic microbeads (Samsel *et al.*, 2011; Bunker *et al.*, 2015; Ito *et al.*, 2016).

The purpose of this chapter is to describe the key elements of experimental protocols to induce and maintain OHT in mice using magnetic microbeads (Samsel *et al.*, 2011; Bunker *et al.*, 2015; Ito *et al.*, 2016) injected into the anterior chamber of the mouse eye and directing their accumulation into the trabecular meshwork to prevent aqueous humor (AQH) outflow. These procedures permit the raising and maintenance of elevated IOP for up to six weeks in order to investigate the effects of chronic OHT on the structure and function of the retina and optic nerve. Such mouse and rat models also provide platforms for testing the ability of pharmacological agents to reduce the IOP and/or protect the retinal ganglion cells (RGCs) and reduce damage to their axons that form the optic nerve. Some representative data obtained from this model of OHT/glaucoma are presented to illustrate the type of information that can be obtained from such studies with a focus on the methods described by Ito *et al.* (2016).

EXPERIMENTAL PROTOCOLS

The following procedures should be performed in compliance with the guidelines for the Use of Experimental Animals and the Statement for the Use of Animals in Ophthalmic and Visual Research from the Association for Research in Vision and Ophthalmology (ARVO). The majority of the procedures described herein are adapted from Ito *et al.* (2016). Alternative methods are also available (Bunker *et al.*, 2015).

Glass Microneedle Preparation for Intracameral Injection of the Microbeads

Whilst glass capillary-based microneedles are used in the current protocol (Ito *et al.*, 2061), it is possible to use sterile 33 G beveled metal needles (Bunker *et al.*, 2015).

- First, create borosilicate glass capillary microneedles using standard glass-pulling techniques
- At an angle under a microscope, use a sharp metal blade to carefully generate an elliptical opening (approx 190 μm and 70 μm) at microneedle tips. Using distilled water, the uniformly created microneedles are then beveled at a 20^{o} angle (relative to the plate in the beveling system) for up to 10 min to ensure that

the final products are smooth and without rough edges or surfaces and, of course, still with sharp ends.
- An aerosol duster is then used to remove any debris on and in the microneedle tips.
- For quality control purposes, ensure that all beveled microneedles are clean and unfractured and then sterilized with ethanol and then with a sterile balanced salt solution (BSS). Allow the microneedles to air dry in a sterile cell culture hood.

Magnetic Microbead Suspension Preparation

Removal of Epoxy Groups from Magnetic Beads

- The epoxy groups coated on the magnetic microbeads need to be removed first before proceeding to the animal work.
- Suspend the magnetic microbeads (MMBs; 4.5 μm diameter, 4 x 10^8 beads/ml) in a solution of 0.02 M sodium hydroxide made up of 10x Tris buffer. Gently vortex the stock of MMBs suspension until the beads are uniformly dispersed and suspended in solution.
- Rapidly remove 1 ml of this suspension and add it to 50 ml of 0.02 M NaOH in 10x Tris buffer. Allow this tube to rotate for 1 day at room temperature in order to free the beads off the epoxy groups.
- Using a magnet, collect the beads at the bottom of the tube and gently remove the supernatant. Re-suspend the beads in fresh 50 ml of 0.02 M NaOH in 10x Tris buffer, gently disperse, and repeat the tube rotation for another 4 hrs as above.
- Using a magnet, collect the beads at the bottom of the tube and gently remove the supernatant without disturbing the "MMBs pellet".
- Gently vortex the pellet in 50 ml of 10x Tris-buffer until the beads are well suspended, and repeat steps 5 & 6 as above.

Concentration and Resuspension of MMBs in Sterile Balanced Salt Solution

- Now concentrate the MMBs in sterile BSS to obtain a final concentration of 1.6 x 10^6 beads/μl.
- Suspend the concentrated MMBs in 5 ml of ultra-pure laboratory-grade water by gently mixing for 2 min on a vortex. Then, concentrate them to the bottom of the tube with a magnet as before and remove the supernatant.
- Repeat the MMBs washing procedure described above an additional 3 times and obtain the washed MMB pellets.
- Under sterile conditions in a laminar flow hood, wash the beads with 0.5 ml of sterile BSS by gentle trituration (pipetting up and down a few times).

CHAPTER 27

Protocol for the Rat Episcleral Vein Cauterization Model of OHT/Glaucoma

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Abstract: Glaucoma caused by ocular hypertension (OHT) poses a grave health problem as our society's demographics tilt towards an ever-increasing aged population. In order to study this ocular disease, several different models of OHT involving a variety of different animal species have been developed over the years. In the current chapter, we will focus on a method to raise intraocular pressure (IOP) by cauterizing two or three trunks of episcleral veins in the eye of rats to induce OHT.

Keywords: Eye, Glaucoma, Intraocular pressure, Ocular hypertension, Protocol, Retina, Retinal ganglion cells, Tonometry, Uveitis.

INTRODUCTION

A rise in intraocular pressure (IOP) occurs when the balance between aqueous humor formation and outflow resistance is compromised, resulting in ocular hypertension (OHT). Long-term elevated eye pressure is a critical risk factor for glaucoma and the leading cause of blindness in the world (Jayanetti *et al*., 2020). Glaucoma is a neurodegenerative disease that has hallmark features that involve loss of retinal ganglion cells (RGCs), thinning of the optic nerve, and the resultant visual field loss affecting mostly the peripheral vision at the beginning, which progressively worsens over time (Bai *et al*., 2014). Over the last two decades, researchers have established and validated numerous animal models that mimic clinical features of OHT and glaucomatous optic neuropathy. Some examples include the hypertonic saline episcleral vein injection model (Mittag *et al*., 2000),

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the laser-induced OHT model (Fu *et al*., 2010), the trabecular meshwork-occlusion with microspheres model (Sappington *et al*., 2010), and spontaneous or genetically-created mouse glaucoma models (Harada *et al*., 2019). The episcleral vein cauterization rat model of OHT involves obstructing two or three trunks of episcleral veins. This rat model of OHT is beginning to be utilized in OHT/glaucoma research since it is quite reproducible and relatively cheap. The purpose of this chapter is to provide a series of steps that can serve as a guide for researchers in the utilization of *in vivo* models of glaucoma in glaucoma research to help discover new etiological factors related to OHT/glaucoma development and for discovering new therapeutic modalities.

EXPERIMENTAL PROTOCOLS

The following procedure was adapted from methods similar to that of Shi *et al.* (2007) and Bai *et al.* (2014). The detailed protocol should be approved by and performed according to guidance provided by the Association for Research in Vision and Ophthalmology (ARVO) "Use of Animals in Ophthalmic and Vision Research" and the Institutional Animal Care and Use Committee (IACUC).

Preparation of the Animals

- Female Wistar Hannover rats (225-250 gm) should be kept in a 12-hour dark-light cycle paradigm and provided food and water ad libitum.
- Use a cocktail of ketamine, xylazine, and acepromazine (injected intraperitoneally at a dose ratio of 50/5/1 mg/kg) to deeply anesthetize the rats on the day of the surgery.

Induction of OHT / Glaucoma

- In the anesthetized rats, make an incision in the conjunctiva followed by isolation of extra-ocular muscles and identification of major limbal drainage veins.
- Cauterize two dorsal episcleral veins and one temporal episcleral vein using a 30″ cautery tip.
- Observe the morphology of the episcleral veins before and after cauterization to ensure no bleeding is present. Use a small amount of a broad topical antibiotic ointment to prevent any future infection.
- Use the contralateral eye with sham surgery as a control.

IOP Measurement

- For IOP measurement, anesthetize the treated rats using light anesthesia consisting of oxygen and a 3% isoflurane mixture (1 liter/min).

- Measure and record the IOPs using a Tono-Pen-XL Applanation tonometer per the manufacturer's instructions.
- Take baseline measurements immediately after cauterization and then every week after the surgery until the rats are sacrificed.
- Each measurement should be repeated at least 3 times, recorded, and later averaged for each time point. IOP readings should be performed at the same time every day or every week as deemed fit.
- The effect of test drugs topically applied to the eye can be determined when the IOP has reached maximum levels. Again, multiple readings should be taken post-drug application over time to determine the time course of IOP changes in the absence and presence of the test compound.

Retinal Thickness Measurements

- Under full anesthesia, apply a topical pupil dilation solution (atropine sulfate 1%, Alcon, USA) to the cornea/conjunctiva.
- Acquire images of retinas from experimental rats using a non-invasive prototype optical coherence tomography (OCT) system.
- Scan 2-4-volumes in each eye in different quadrants of the retina.
- For each volume, take six high-quality frames and take four measurements in one frame adjacent to 1 mm from the optic nerve head.
- Average the four recordings as the thickness of each frame.
- The OCT measurements need to be performed before and after the cauterization procedure to detect changes in the retinal thickness.

Retinal Function Determination

- Following 15 hours of adaptation following the vein cauterization surgery, rats should be anesthetized and kept under dim red illumination light ($\lambda > 600$ nm).
- Now, dilate the pupil using topical atropine treatment.
- Place the gold ring electrode of the electroretinogram (ERG) system over the central part of the corneal surface.
- Place a reference electrode and a ground electrode onto the ear and in the tail, respectively.
- Now, take ERG measurements simultaneously from both eyes. Later, using a photic stimulator (Ganzfeld Q400, USA), record at least 10 retinal responses to flashes of unattenuated white light (5 ms, 0.2 Hz).

CONCLUSION

The rat episcleral cauterization protocol described herein has been developed and validated in several studies. It is a fairly cheap, reproducible, and reliable animal model of OHT, which can be an asset for studying factors involved in

CHAPTER 28

Protocol for Silicone Oil-Induced Ocular Hypertension

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Abstract: This chapter describes the protocols to induce ocular hypertension in rabbits using silicone oil. This process consists of many steps and sub-steps, including preparation of the animal for anesthesia, preparation of materials, administration of anesthesia, preparation for silicone oil (SO) injection, intracameral injection of SO, and finally, postoperative care. Healthy animals free of ocular morphological abnormalities or disease states should be chosen for inclusion before beginning the protocol. Following these steps will allow for the simulation of ocular hypertension while also ensuring an aseptic environment.

Keywords: Eye, Glaucoma, Intraocular pressure, Ocular hypertension, Protocol, Retinal ganglion cells, Silicone oil.

INTRODUCTION

Changes to aqueous humor (AH) flow can have a significant impact on visual acuity in both humans and animals. In the normal function of the eye, AH must flow through the anterior chamber to drain through the trabecular meshwork (TM); however, if this pathway is altered, pressure differentials can occur (Zhang *et al*., 2019). If AH outflow through the TM is excessive, this can cause intraocular pressure (IOP) to decrease, and uveitis may occur. Conversely, if AH outflow is obstructed, IOP may increase, potentially leading to glaucoma and damage to the retinal ganglion cells (RCGs) (Zhang *et al*., 2019; Ofri, 2020).

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Animal models can be useful not only for understanding the pathophysiology of naturally occurring ocular disease but also for presenting models of disease that may be modified by researchers to create targeted drug therapies. One such animal model is the rabbit. At a comfortable baseline, rabbits typically produce IOP values in the range of 15-23 mm Hg (Vareilles *et al*., 1977). Instead of waiting years for the possible occurrence of natural glaucoma in these animals, it is possible to simulate glaucoma using an intracameral injection of silicone oil (SO) (Zhang *et al*., 2019). The purpose of this chapter is to describe a veterinary protocol for the artificial induction of ocular hypertension in rabbits. This will allow researchers to simulate the glaucomatous disease state so that they may evaluate new drug therapies for those afflicted.

EXPERIMENTAL PROTOCOLS

The following procedure should be performed in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology (ARVO).

All testing should be performed on healthy rabbits. Those with a history of prior ocular disorders or morphological changes to the cornea should be excluded from this study.

Preparation of the Animal for Anesthesia

NOTE: There is no need for the animals to be placed on a fast before surgery, as rabbits are unable to vomit (Horn *et al*., 2013).

- Using a scale, obtain an accurate weight in kilograms for the rabbit.
- Place the rabbit gently on a heating pad on top of the examination table.
- Gently restrain the rabbit by wrapping it in a towel.
- Shave a small area on the back of the rabbit's neck to prepare for subcutaneous injection.

Preparation of Materials

- Attach a sterile 25 G needle to a sterile plastic 3 mL Luer Lock syringe. Ensure that the needle is screwed on tightly.
- Use a 70% alcohol wipe to sterilize the injection site of a vial of 100 mg/mL ketamine before withdrawing a dose of 10 mg/kg body weight using the syringe prepared.
- Use a 70% alcohol wipe to sterilize the injection site of a vial of 20 mg/mL xylazine before withdrawing a dose of 2 mg/kg body weight using the syringe prepared above.
- Attach a sterile 25 G, 0.5-inch needle to a plastic 1 mL Luer Lock syringe.

Ensure that the needle is screwed on tightly and that the plunger is fully depressed.
- Use a 70% alcohol wipe to sterilize the injection site of a vial of 1,000 centistokes silicone oil (SILIKON®) before using the syringe as above to withdraw 1 mL of silicone oil.
- Prepare a dilute solution (1:50 in sterile normal saline) of betadine (Povidone-Iodine 10%) for pre-surgical irrigation of the eye.

Administration of Anesthesia

- Apply lidocaine cream topically to the area to be injected and wait for a few minutes before administering the anesthetic injection.
- Use a 70% alcohol wipe to sterilize the shaved portion of the rabbit's neck. Allow the alcohol to dry before injection.
- Using the thumb and forefinger of one hand, pinch the shaved skin on the back of the rabbit's neck and pull it away from the body.
- Insert the needle of the syringe containing ketamine into the pinched skin at a 45-degree angle and depress the plunger completely.
- Discard the used syringe and needle in a Sharps container.
- Repeat steps for the xylazine syringe.
- As sedation sets in, place the animal on heat support and maintain ½" of padding/material between the animal and the heat source.
- About 7-10 minutes following injection of anesthetic agents, place a face mask on the rabbit to administer oxygen mixed with 1% isoflurane initially. Isoflurane concentration may be increased up to 5% to maintain sedation.
- Pinch the rabbit's toe to monitor for degree of sedation and to determine if an additional sedation is required. If no whisker or tail movement is noted, sedation is sufficient.

NOTE: Once anesthesia has been administered, monitor the rabbit's heart rate, respiratory rate, and temperature continuously or every 5 minutes as resources allow.

Preparation for SO Administration

- While on the heat support/padding, place the rabbit on one side, in a lateral recumbent position, with the desired eye facing upward.
- Use two fingers of one hand to hold open the eye planned for injection.
- Using the other hand, irrigate animal eyes with 1:50 betadine/sterile water or sterile saline solution.
- Then, with the same hand, instill one drop of 0.5% proparacaine hydrochloride into the eye to coat the cornea.
- Wait 1-2 minutes to allow for absorption of proparacaine.

CHAPTER 29

Protocol for Inducing Ocular Hypertension in Rats and Mice Using Circumlimbal Sutures

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Abstract: The circumlimbal suture technique for inducing experimental ocular hypertension (OHT) in rodents can be utilized to achieve chronic intraocular pressure (IOP) elevation, however, it is fully reversible. This protocol provides guidance for the application and adjustments of the suturing approach for both rats and mice. In brief, by placing the animals under general anesthesia, a "purse-string" suture is threaded through and anchored to the conjunctiva around the equator, behind and parallel to the eyeball limbus. Using this procedure, moderate levels of IOP increases can be achieved over a 12-week time period. This protocol can induce retinal dysfunctions, specifically both structural and functional, commensurate with retinal ganglion cell demise and optic nerve damage. Removal of the suture and restoration of IOP back to baseline levels afford functional recovery when the intervention is early. This circumlimbal suture procedure is a minimally invasive and cost-effective means to induce OHT that reliably results in RGC and optic nerve injury in rodents. The effects of potential IOP-lowering and neuroprotectants can be assessed in this novel animal model of OHT.

Keywords: Circumlimbal suture, Eye, Glaucoma, Intraocular pressure, Ocular hypertension, Optic nerve, Protocol, Retinal ganglion cells.

INTRODUCTION

Development and validation of suitable animal models are important to study neurodegeneration due to ocular hypertension (OHT)/glaucoma. Many inducible models have been established to produce long-term increases in intraocular pressure (IOP), the most important risk factor for the development of glaucoma. These approaches induce OHT (Bunker *et al.* 2015; Chiu *et al.* 2007; Feng *et al.* 2013; Moreno *et al.* 2005; Morrison *et al.* 1997; Quigley and Addicks 1980;

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Weber and Zelenack 2001), with the goal of recapitulating specific and progressive injury to retinal ganglion cells (RGCs), with minimal complications such as trauma and inflammation.

In this chapter, we describe a step-by-step protocol for inducing chronic IOP elevation in rats (Lakshmanan *et al.* 2020; Liu *et al.* 2015) and mice (Zhao *et al.* 2017a) using a circumlimbal suture, which increases episcleral venous pressure (Wong *et al.* 2021) resulting in sustained IOP elevation. One important feature of this model is the reversibility of the IOP to baseline by simply removing the suture. IOP normalization may be useful for studying the cellular and molecular correlates of reversible and irreversible RGC injury (Liu *et al.* 2017; Zhao *et al.* 2019).

EXPERIMENTAL PROTOCOLS

All experimental procedures were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, set by the National Health and Medical Research Council in Australia, as well as the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology (ARVO).

Intraocular Pressure Measurement in Conscious Rodents

Note: To obtain robust baseline IOP, measurements can be undertaken for several days (*e.g.*, 3 -5 days) prior to surgery to acclimatize animals to the procedure. Measurements can be taken using a calibrated rebound tonometer with or without a topical drop of proparacaine hydrochloride. For longitudinal monitoring, the IOP should be measured at about the same time of day and under the same lighting conditions for consistency (Aihara *et al*., 2003; Jia *et al*., 2000).

- Set the rebound tonometer to the correct calibration (rat or mice) as per the manufacturer's instructions.
- For ease of handling of rats, try to wrap the awake rat in a soft cloth and use gentle rocking motion to calm the animal. Gently expose the head and neck and carefully hold the torso in one hand, with the rat's back resting against the researcher's chest.
- For mice, hold the animal by the scruff of the neck and hold the tail between the ring finger and middle finger (or between the little finger and your palm). Avoid too much pressure on the skin so that little impact occurs on the eyes and potential suffocation is prevented.
- When using a rebound tonometer, use the other hand to align the tonometer so that the tip of the IOP probe is approximately 2 – 3 mm away from and perpen-

dicular to the corneal apex. Press the measurement button whilst ensuring that the tip of the probe contacts the corneal apex.

Note: A single beep of the tonometer confirms successful measurement, which can be read from the LCD screen. A measurement error is indicated by a dual beep. Such errors arise due to many factors, including excessive tilt, incorrect working distance, the probe striking the periphery of the cornea, and the touching of eyelashes or eyelids. Careful reading and executing the guidelines for the use of the rebound tonometer should reduce the potential measurement errors.

- Record individual reading for each of ten repeats, each at an interval of 1 – 2 seconds. Reset the tonometer after the 5th reading.

Note: If possible have another investigator take the IOP readings to ensure correct reproducible data are collected.

Induction of Intraocular Pressure Elevation in Anesthetized Rodents

- Autoclave all surgical equipment beforehand.
- Clean the surgical bench with disinfectant (*e.g.*, 0.5% chlorhexidine in 70% ethanol).
- Use sterile drapes to cover the bench.
- Appropriate personal protective equipment should be worn by all research staff (*e.g.*, sterilized gloves, gowns, and masks).
- Anaesthetize animals with 3 – 3.5% isoflurane with O_2 at a flow rate of 3 L/min (flow rate is usually the same as the size of the induction container) and then maintain using 1.5% isoflurane at 0.5 - 1 L/min. Place the animal on a heat pad to maintain body temperature during surgery. Adjust the isoflurane level to ensure sufficient surgical plane of anesthesia. Monitor and adjust the flow of the anesthetic to obtain about 60 breaths/min breathing rate.
- Now, select one eye and lightly numb the cornea with a drop of 0.5% proxymetacaine ophthalmic solution.
- Use sterile surgical drapes to cover the animal while leaving the treated eye exposed. To prevent desiccation of the other eye, place some lubricating gel on it.
- Under a microscope, weave a 7/0 nylon suture under (5 – 6 anchor points in rats; 4 – 5 anchor points in mice) and above the conjunctiva parallel and 2 mm posterior to the limbus (Fig. **1**). For mice, use a 10/0 nylon suture at 1 mm posterior to the limbus, avoiding penetration of the sclera. It is critical that compression of the major episcleral veins be avoided by threading the suture underneath the conjunctiva at the crossing of these veins. The episcleral veins are hard to distinguish/see in mice; thus, avoiding them may be somewhat difficult.

CHAPTER 30

Protocol for Corticosteroid-Induced Ocular Hypertension and Glaucomatous Optic Neuropathy in Rodents

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Abstract: Many forms of glaucoma afflict patients around the world and rob them of their eyesight. Secondary glaucoma is induced by many factors, such as trauma to the eye, injury-induced inflammation, pathogen-induced inflammation, and drug-induced pathology of the major aqueous humor outflow system, the trabecular meshwork. When patients experience inflammation and irritation on the ocular surface or within the anterior chamber of the eye (*e.g.*, uveitis), they are prescribed topical ocular formulation of corticosteroid (*e.g.*, dexamethasone) and perhaps also oral anti-inflammatory steroids to reduce the symptoms and calm down the eye. However, a major side effect of such corticosteroid treatment is the development of secondary glaucoma in at least 30% of the population who are susceptible to steroids. In order to find suitable remedies for this condition, a variety of animal models of steroid-induced ocular hypertension (OHT) have been developed for testing drugs and other therapeutics. Some of these models and techniques will be described in this chapter.

Keywords: Animal model, Intraocular pressure, Ocular hypertension, Steroids, Secondary glaucoma.

INTRODUCTION

Ocular inflammation can be caused by many internal and external factors, including microbial infestation, blunt trauma to the eye, and irritation caused by drug eyedrop administration or airborne pollutants and allergens. A common treatment for such conditions is instillation of topical ocular and oral anti-inflammatory steroids such as dexamethasone (DEX). Unfortunately, steroid-induced elevation of intraocular pressure (IOP), ocular hypertension (OHT), and

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glaucoma often result in 20-30% of patients who possess a gene that is switched on by corticosteroids (Stone *et al*., 1997). This gene overproduces a mutated form of an endogenous protein, mutant myocilin, which is released by cells within the major outflow pathway of aqueous humor (AQH) drainage from the anterior chamber of the eye, the trabecular meshwork (TM) (Clark *et al*., 1994, 1995). The chronic obstruction of the TM by accumulated extracellular matrix components such as collagen and fibronectin and the mutated myocilin reduces or halts the drainage of AQH, and this raises the IOP (Stone *et al*., 1995; Weinreb, 2005; Patel *et al*., 2017; Sharif, 2021, 2023). Over time, even acute OHT begins to damage the retinal ganglion cells and the optic nerve, and patients begin to experience visual impairment if the steroidal treatment is not curtailed and the steroid removed from the body. Corticosteroid-stimulated OHT develops within weeks in vulnerable patients. Its onset, severity, and final outcome (degree of vision loss) depend on the steroid potency, its ability to penetrate the cornea and conjunctiva (pharmacokinetics), duration of treatment, and route of administration, with topical ocular (or periocular injection, at least in animals) being the most rapid inducer of the OHT (Clark *et al*., 1995; Zhang *et al*., 2007; Whitlock *et al*., 2010; Zode *et al*., 2011, 2014; Maddineni *et al*., 2020).

In order to better understand the pathophysiology of steroid-induced IOP increase, a number of model systems have been developed that try to capitulate the human condition. These range from ex vivo cell and anterior eye segment organ cultures to *in vivo* animal models using rodents, cattle, and even non-human primates (Rybkin *et al*., 2017; Li *et al*., 2019). These model systems can then be utilized to investigate specific aspects of IOP elevation and to better understand the pathological sequelae involved in disease development and progression, as well as the means to find suitable remedies to overcome the symptoms of the disease and its consequences. Commonly used rodent models of steroid-induced glaucoma will be described in this chapter (Maddineni *et al*., 2020).

From a phylogenetic perspective, rodents are the lowest mammals where IOP increase with steroidal treatment has been reported. Even though rats offer a larger eye for manipulation and perhaps ease of handling, mice are cheaper, and they provide an animal platform that can be genetically altered. As such, three mouse models of steroid-induced OHT are currently available for utility in research. These include the following: 1). Topical ocular steroid treatment model. In this model, DEX eyedrops (0.1%) are instilled in the eyes of normal mice three times daily for 6 weeks (Zode *et al*., 2014). Here, the animals develop OHT starting at 2 weeks after steroid administration, and the IOP can be elevated by up to 7 mmHg from baseline. These mice develop glaucomatous optic neuropathy (GON), where the RGCs and their axons are significantly reduced (Zode *et al*., 2014). 2). Systemic corticosteroid treatment model. Here, mice are subjected to

continuous systemic exposure to DEX, which is delivered *via* osmotic minipumps for up to 1 month (*e.g.*, 0.09 mg DEX/day) (Overby *et al*., 2014; Whitlock *et al*., 2010). Unfortunately, the systemic steroid treatment only elevates the IOP by 2-4 mmHg, and there is high mortality in the mice. 3). Periocular corticosteroid treatment model. Here, triamcinolone acetonide (40mg/ml – 20ul per eye) is given to the mice *via* the periocular route of administration (Maddineni *et al*., 2020). This model shows an IOP increase with reduced TM outflow and GON (Maddineni *et al*., 2020; Sharif *et al*., 2024) and will be described below.

Periocular Administration of DEX to Raise IOP

Animal Preparation

- All experiments are to be performed after receiving and documenting written permission from the Institutional Animal Care and Use Committee (IACUC), and according to the guidelines provided by the Assoc. Research in Vision and Ophthalmology (ARV). At the conclusion of the experiments, mice should be sacrificed by CO_2 inhalation and then by cervical dislocation or as approved by institutional IACUC protocol.
- Use 3-month-old male C57BL/6 J mice (Jackson Laboratory; Bar Harbor, ME), feed rodent/mouse standard chow ad libitum, and maintain them on a 12 hrs light/dark cycle at 21°C-26 °C and with 40-70% humidity.

Periocular Administration of DEX and IOP Measurements

- Prepare DEX suspension by mixing 10 mg of micronized DEX-Acetate (Spectrum Chemicals, New Brunswick, NJ) in 1 mL of vehicle (Veh). A uniform suspension with a desired DEX particle size can be achieved by mixing the suspension along with two stainless steel 5-mm beads (Qiagen, Valencia, CA) in a Tissue Lyser LT (Qiagen) for 10 min at 50 oscillations/sec and further rotated overnight at 4°C.
- Anesthetize the mice with isoflurane (2.5%) and oxygen (0.8 L/min) and then peri-ocularly inject 200 µL/eye of either Veh or freshly made DEX (*i.e*., 200 µg) using a 32-gauge needle attached to a Hamilton glass micro syringe. This procedure should be repeated on a weekly basis for up to 6-8 weeks to raise the IOP. For most experiments, both eyes should be injected with either vehicle alone or DEX (*i.e*., bilaterally). To study axonal transport, one eye should be injected with the vehicle while the contralateral eye should receive the DEX.
- Anesthetize mice as above to measure their IOPs using a TonoLab rebound tonometer (Colonial Medical Supply, Franconia, NH). Day and night-time IOPs should be recorded once a week throughout the treatment period. IOP measurements should be obtained in a masked manner, and an average of six IOP readouts should be taken at each time point. Additionally, dark-adapted

CHAPTER 31

Protocol for Measuring Intraocular Pressure in Guinea Pigs

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Abstract: This chapter describes the protocols to measure intraocular pressure (IOP) in guinea pigs. The process consists of many steps and sub-steps, including the preparation of the animal, the preparation of the TonoVet-Plus® rebound tonometer, and finally, the measurement of IOP itself. Healthy animals free of ocular morphological abnormalities or disease states should be chosen for inclusion before beginning the protocol. Following this series of steps will ensure that IOP readings are taken accurately and with precision.

Keywords: Eye, Glaucoma, Intraocular pressure, Protocol, Retina, Retinal ganglion cells, Tonometry, Uveitis.

INTRODUCTION

Ocular disorders can have a significant impact on visual acuity in both human and animal populations. Changes in intraocular pressure (IOP) can cause lasting damage to the retina and adjoining tissues of the eye. In guinea pigs, for example, there is a wide range of normal IOP values; however, previous studies on normal, healthy guinea pigs found average IOP values ranging anywhere from 9 to 23 mm Hg, concentrating primarily around 15-19 mm Hg (Coster *et al*., 2008; Di *et al*., 2017; Wu *et al*., 2020). IOP readings higher than the typically observed range of values indicate an increased risk for glaucoma, in which aqueous humor (AH) outflow is obstructed, leading to lasting damage to the retinal ganglion cells (RGCs) (Ofri, 2021; Gelatt, 2008). Lower than expected IOP values may indicate excess AH outflow, putting the patient at risk for uveitis (Ofri, 2021). For these reasons, careful monitoring and screening protocols are needed to ensure that these disease states are caught early so that they can be managed appropriately.

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Najam A. Sharif and Sunny E. Ohia (Eds.)

The purpose of this chapter is to describe a veterinary protocol for the evaluation of IOP in guinea pigs. This will provide useful insight into the applicability of animal models for research into new drug therapies that may one day be used in human patients. It will allow for further establishment of baseline expected IOP values for these animals to detect any deviations from the norm, which may be targeted by drug therapies.

EXPERIMENTAL PROTOCOLS

The following procedure was performed in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology (ARVO).

All testing should be performed on healthy guinea pigs. Those with a history of prior ocular disorders or morphological changes to the cornea should be excluded from this study.

Preparation of the Animal for Evaluation

- Gently place the guinea pig in a comfortable sternal recumbent position.
- Allow the guinea pig about five minutes to acclimate to this position. Do not begin testing if the animal is in distress or if excess force is needed to secure the animal in this position.

Preparation of the TonoVet-Plus®

Note: TonoVet-Plus® rebound tonometer has been shown to be more accurate than TonoVet® at measuring IOP when values exceed 14 mm Hg; however, if all values are expected to be below this threshold, TonoVet-Plus® or TonoVet® may be used interchangeably (Shim *et al*., 2020).

- Press the Select or Measure button on the tonometer to turn it on.
- Remove the red cap from the probe base.
- Remove the cap from the tube holding the probe and use the tube to insert the probe into the probe base. Do not touch the probe itself.
- A “play” symbol and species setting should appear on the tonometer’s display, indicating that it is ready for use.

Species Selection

Note: Previous IOP studies have been done on guinea pigs using the TonoVet-Plus® in the “Rabbit” setting due to the animals’ similarity in size (Wu *et al*., 2020). Normal IOPs for rabbits range from 15-23 mm Hg, which encompasses the range of expected values for guinea pigs (Vareilles *et al.* 1977). The TonoVet-

Plus® may be further calibrated in the "p" setting if IOP values are expected to differ from species pre-sets (Icare, 2015). Refer to the manufacturer's website for more information (tonovet.com).

- Press the select button on the tonometer.
- Use the Navigation buttons to find Setting, and then press Select again.
- Use the Navigation buttons to toggle through species until "Rabbit" appears, and then press Select.

IOP Measurement

Note: A study using the TonoVet® to measure differences in IOP based on probe distance from the eye found that the farther the probe was from the eye, the slower the probe speed and the higher the observed IOPs. The newer model tonometer is purported to have a faster probe speed to minimize this concern. As such, the manufacturer recommends holding the probe at 4-8 mm from the eye, with the range in distance accounting for human error (Rodrigues, 2021; Icare, 2015).

- Instill one drop of proparacaine hydrochloride (0.5%) into each animal's eye.
- Position one hand with fingers both above and below the left eye to hold the eyelids open.
- With the other hand, position the TonoVet-Plus® so that the probe tip is horizontal and perpendicular to the cornea, at a distance of 4-8 mm from the corneal surface. A green probe light indicates that the horizontal positioning is correct. If this light is red, the machine is being tilted vertically, and placement should be adjusted. If this light is blinking, press the Measure button to clear this error signal.
- Hold down the Measure button to initiate the collection of six consecutive measurements. The TonoVet-Plus® will automatically discard the highest and lowest readings and project an average of the remaining four IOP readings onto the display.
- If the ring surrounding the final IOP reading is yellow or red, repeat the sequence, as this will indicate that the standard deviation is greater than the accepted 5% (Gloe *et al.* 2019).
- Record the resulting IOP measurement.
- Repeat steps for the right eye.
- Discard the probe after use. The same probe may be used for both eyes in a single guinea pig as long as neither eye is infected.
- To turn off the tonometer, press and hold the Select button until the power sign appears. The device will also turn off automatically after three minutes of use.

CHAPTER 32

Protocol for Measuring Intraocular Pressure in Dogs

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Abstract: This chapter describe the protocols to measure intraocular pressure (IOP) in canines. The process consists of many steps and sub-steps, including the preparation of the animal, the preparation of the TonoVet-Plus® rebound tonometer, and finally, the measurement of IOP itself. Healthy animals free of ocular morphological abnormalities or disease states should be chosen for inclusion before beginning the protocol. Following this series of steps will ensure that IOP readings are taken accurately and with precision.

Keywords: Eye, Glaucoma, Intraocular pressure, Protocol, Retina, Retinal ganglion cells, Tonometry, Uveitis.

INTRODUCTION

Like humans, ocular disorders are prevalent in the canine population. Changes in intraocular pressure (IOP) cause etiologies, which can lead to lasting damage to the retina. In dogs, for example, there is a wide range of normal IOP values, which may vary based on breed; however, 15 to 25 mm Hg is standard (Kulualp *et al*., 2017; Ofri, 2021). IOP readings higher than the accepted range of values put the animal at risk for the development of glaucoma, in which the increased pressure contributes to the obstruction of aqueous humor (AH) outflow. This, in turn, can cause permanent damage to retinal ganglion cells (RCGs) (Ofri, 2021; Gelatt, 2008). Conversely, IOP values that are lower than expected are often associated with uveitis and can be attributed to excess AH outflow. When screening for these disease states, both eyes should have similar pressures within 10 mm Hg of each other. Any larger deviations may be suspicious for the development of glaucoma (Ofri, 2021).

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The purpose of this chapter is to describe a veterinary protocol for the evaluation of IOP in canines. Animal models of eye disease provide valuable insight into identifying the potential factors underlying ocular disorders. Additionally, animal models present a useful tool for the evaluation of the *in vivo* pharmacological efficacy of drugs that may help treat eye conditions in both humans and animals. This protocol allows for the detection of any deviation from standard IOP values and, by extension, allows screening for potential ocular disease states, which may then be targeted by drug therapies.

EXPERIMENTAL PROTOCOLS

The following procedure should be performed in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology (ARVO).

All testing should be performed on healthy, conscious dogs. Those with a history of prior ocular disorders or morphological changes to the cornea should be excluded from this study.

Preparation of the Animal for Evaluation

- Gently place the dog in a comfortable sternal recumbent position.
- Allow the dog about five minutes to acclimate to this position. Do not begin testing if the animal is in distress or if excess force is needed to secure the animal in this position.

Preparation of the TonoVet-Plus®

Note: TonoVet-Plus® rebound tonometer has been shown to be more accurate than TonoVet® at measuring IOP when values exceed 14 mm Hg; however, if all values are expected to be below this threshold, TonoVet-Plus® or TonoVet® may be used interchangeably (Shim *et al*., 2020).

- Press the Select or Measure button on the tonometer to turn it on.
- Remove the red cap from the probe base.
- Remove the cap from the tube holding the probe and use the tube to insert the probe into the probe base. Do not touch the probe itself.
- A “play” symbol and species setting should appear on the tonometer’s display, indicating that it is ready for use.

Species Selection

- If the species shown on the display is incorrect, press the Select button.
- Use the Navigation buttons to find Setting, and then press Select again.

- Use the Navigation buttons to toggle through species until "Canine" appears, and then press Select.

IOP Measurement

Note: A study using the TonoVet® to measure differences in IOP based on probe distance from the eye found that the farther the probe was from the eye, the slower the probe speed and the higher the observed IOPs. The newer model tonometer is purported to have a faster probe speed to minimize this concern. As such, the manufacturer recommends holding the probe at 4-8 mm from the eye, with the range in distance accounting for human error (Rodrigues, 2021; Icare, 2015).

- Instill one drop of proparacaine hydrochloride (0.5%) into each animal's eye.
- Position one hand with fingers both above and below the left eye to hold the eyelids open.
- With the other hand, position the TonoVet-Plus® so that the probe tip is horizontal and perpendicular to the cornea, at 4-8 mm from the corneal surface. A green probe light indicates that the horizontal positioning is correct. If this light is red, the machine is tilted vertically, and placement should be adjusted. If this light blinks, press the Measure button to clear this error signal.
- Hold down the Measure button to initiate the collection of six consecutive measurements. The TonoVet-Plus® will automatically discard the highest and lowest readings and project an average of the remaining four IOP readings onto the display.
- If the ring surrounding the final IOP reading is yellow or red, repeat the sequence, as this will indicate that the standard deviation is greater than the accepted 5% (Gloe *et al.* 2019).
- Record the resulting IOP measurement.
- Repeat steps 3.1-3.5 for the right eye.
- Discard the probe after use. The same probe may be used for both eyes in a single dog as long as neither eye is infected.
- To turn off the tonometer, press and hold the Select button until the power sign appears. The device will also turn off automatically after three minutes of use.

CONCLUSION

The protocol described here is sufficient for anyone in possession of a TonoVet-Plus® rebound tonometer who can repeat this procedure to measure IOP in canine subjects. It does not contain an exhaustive report of all error messages that may be encountered. For this reason, readers are encouraged to visit the manufacturer's website (tonovet.com) for any additional inquiries.

CHAPTER 33

Laser Model of Ocular Hypertension in Nonhuman Primates

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Abstract: This chapter describes a protocol to generate a chronic model of elevated intraocular pressure in nonhuman primates. The main piece of equipment is a good laser designed for use in humans but dedicated exclusively for NHP use. The preoperative procedures include anesthesia, which should be done by certified trained personnel. The actual laser procedure only takes a few minutes, preferably by an ophthalmologist. Forty spots are administered to one eye around 8 clock hours of the trabecular meshwork (80 spots). The fellow eye remains untouched. The animal is allowed to recover for one month, during which time it is monitored daily. Intraocular pressures (IOPs) are taken every few days. The laser procedure is repeated at one month (30 spots) and possibly two months (15 spots) depending on the rise in pressure. The contralateral eye is never lasered. Recovery from the last laser procedure requires three to six months, after which time the animal is ready for study. The IOPs of the lasered eye will be more variable than the contralateral eye. A successful procedure yields unilateral ocular hypertension that persists for years.

Keywords: Argon laser, Glaucoma, Intraocular pressure, Ocular hypertension, Trabecular meshwork, YAG laser.

INTRODUCTION

The nonhuman primate (NHP) is a valuable model used to study glaucoma because of its similarity to humans in its anatomy, physiology, and response to intraocular pressure (IOP)-lowering treatments. Laser treatment to the trabecular meshwork can cause a chronic elevation in IOP that becomes glaucoma. The model was developed in the 1980s (Quigley and Hohman, 1983) and has been

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used over the past four decades in studies on IOP effects on aqueous humor dynamics (Toris *et al*., 2000), optic nerve head ischemia (Bellezza *et al*., 2003), electroretinograms (Marx *et al*., 1986), progression of cupping (Pederson and Gaasterland, 1984), changes in visual fields (Sasaoka *et al*., 2008), morphological changes in outflow pathways (Sasaoka *et al*., 2008, Zhang *et al*., 2009), prescribed IOP lowering drugs (Lee *et al*., 1985, Podos *et al*., 1989), and IOP lowering drugs in preclinical studies (Bastia *et al*., 2021a, Bastia *et al*., 2021b, Fuwa *et al*., 2018a, May *et al* ., 2003, Toris *et al*., 2005, Toris *et al*., 2006).

The protocols used today in our lab are modifications of the original procedures from the 1980s. Three main changes have been incorporated. First, the laser procedure is less aggressive than the original method. Laser is done over two to three sessions with a month's recovery between treatments. Second, 10-15 degrees of the trabecular meshwork are always left untouched. This avoids excessive and painful extremes in IOP that reduce the usefulness of the model. Third, the laser treatment avoids the iris root so as not to cause a permanently dilated and asymmetric pupil. Below are details of our protocol for treating chronic glaucoma in NHPs.

EXPERIMENTAL PROTOCOLS

The following procedures were performed with the approval of the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conformed to the Statement for the Use of Animals in Ophthalmic and Visual Research from the Association for Research in Vision and Ophthalmology (ARVO).

Outline of Procedures

Animals

i. Species most often used
   - *Macaca fascicularis* (cynomolgus macaques)
   - *Macaca mulatta* (Rhesus macaques)

ii. Number of animals
   - For IOP studies, 6 to 8 animals per group
   - For studies of aqueous humor dynamics, 12-16 animals per group
   - For morphological studies, 3-5 animals per group

Equipment

i. Neodymium-doped YAG diode laser or Argon Ophthalmic laser with adjustable table and slit lamp (Alcon, Ft. Worth, TX). The laser should be dedicated to animal use only. It should not be shared with humans.

Supplies

i. Personal Protective Equipment (PPEs); a set for each person handling or near animals
   - Kevlar gloves for handling awake animals
   - Surgical gloves for all other procedures
   - Coveralls
   - Mask
   - Face shield
   - Shoe covers
   - Hair net

ii. Prep area
   - Absorbent pads to cover surfaces that animals could contaminate
   - Sterile drapes to cover areas near the eye
   - Heating pad
   - Disinfectant
   - Soft tissue wipes

iii. Anesthesia
   - Isoflurane anesthesia delivery system
   - Isotec (isoflurane) gas canister
   - O_2 tank
   - Respirator
   - Laryngoscope
   - Endotracheal tube
   - Gauze tie
   - Syringe for inflating balloon
   - Surgical tape
   - Needles
   - Syringes
   - Scissors

iv. Drugs
   - Ketamine, for the safe handling of animals
   - Cetacaine for gag reflex suppression
   - Banamine (flunixin meglumine) or other pain reliever
   - Proparacaine hydrochloride, 0.5%, to anesthetize the cornea
   - Artificial tears, such as EyeStream (Alcon) to keep the cornea hydrated

CHAPTER 34

Protocol for Assessment of Aqueous Humor Dynamics in the Living Mouse

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Abstract: A protocol is described in detail to make an assessment in a mouse model in an experimental session of all principle parameters of aqueous humor dynamics (AHD), specifically intraocular pressure (IOP), aqueous flow rate (F_{in}), uveoscleral outflow rate (F_u), total aqueous outflow facility (C_{tot}), and episcleral venous pressure (EVP). Using the technique described, direct measurements of IOP, C_{tot}, and EVP can be made. On the basis of these measurements, by utilizing the modified Goldmann equation, values for F_{in} and F_u can then be derived. These measurements have relevance in the mouse, as the anterior segment of the mouse eye has many anatomic, physiologic, and pharmacologic features in common with the human eye. The mouse has found much use over the last two decades as an animal model for the study of primary open-angle glaucoma (POAG), a progressive optic neuropathy and a leading cause of irreversible blindness globally. POAG is very often associated with elevated IOP and disturbances in other parameters of AHD. Thus, an ability to quantify these parameters in this species has much relevance to research into POAG, especially when considering (1) animal model validation, and (2) effects of experimental compounds designed to modify AHD.

Keywords: Anterior chamber, Aqueous flow, Aqueous humor dynamics, Aqueous outflow facility, Eye, Episcleral venous pressure, Glaucoma, Intraocular pressure, Mouse, Posterior chamber, Protocol, Uveoscleral outflow.

INTRODUCTION

Glaucomas are a group of optic neuropathies in which there is a progressive loss of retinal ganglion cell somas and their axons, as well as damage to the optic nerve and certain centers involved in the relay of visual impulses in the brain. Primary open-angle glaucoma (POAG) accounts for ~86% of all glaucomas in the

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world (Tham *et al.,* 2014). It is estimated that by 2040, there will be 111,800,000 cases of glaucoma globally, an increase of ~47% since 2020 (Tham *et al.,* 2014). In the USA alone, 150,000 people have been bilaterally blinded by the condition. In POAG, there is pathological damage to the trabecular meshwork (TM), which forms part of the principal outflow pathway for aqueous humor. This damage, which often develops slowly over a period of many years, involves endoplasmic reticulum (ER) stress in TM cells, as well as increased synthesis and deposition of the insoluble structural proteins, fibronectin, laminin, elastin, and collagen types I, III, IV, and VI, as well as increased expression of various glycosaminoglycans and the cross-linking enzymes transglutaminase and lysyl oxidase (Kasetti *et al.,* 2017; Raychaudhuri, Millar & Clark, 2017). This leads to a gradually increased resistance beyond normal values to aqueous humor outflow *via* this route. Since the eye is a closed system, the pressure required to drive the aqueous out of the anterior chamber often slowly increases. Thus, the intraocular pressure (IOP) is often increased from equilibrated values of 10 to 15 mmHg (healthy eye) to a higher equilibrated value in excess of 21 mmHg (after POAG has developed over several years). It does still hold true, however, that at equilibrated IOP, whatever its value, the total aqueous humor secretion rate must be equal to the total aqueous humor outflow rate. If the former exceeds the latter, IOP will continue to rise until the increased total outflow rate with increasing IOP finally equals the total inflow rate, and then IOP stabilizes at a new, higher value. If the former is less than the latter, then IOP will drop until the reduction in total outflow rate with dropping IOP finally equals the total inflow rate, and then IOP stabilizes at a new, lower value. Indeed, this positive correlation between changes in total aqueous humor outflow rate with corresponding changes in IOP is what constitutes total aqueous outflow conductance or facility (represented by $C_{(tot)}$, mathematically the reciprocal of total aqueous humor outflow resistance). Healthy eyes or eyes with POAG exhibit higher or lower values of $C_{(tot)}$, respectively.

This situation is qualitatively different from the situation seen in complete 360° angle closure, whereby all of the TM is completely covered by the peripheral iris, and aqueous humor cannot access the trabecular outflow pathway. In such cases, no amount of increase in IOP will increase the trabecular outflow rate of aqueous humor (which is zero), and thus, IOP will spike very rapidly (within a matter of a few hours) to values of around 50 to 100 mmHg, or sometimes even greater. In such cases, the alternative aqueous humor outflow pathway, the uveoscleral pathway, which exhibits a very minimal capacity for increase in outflow rate with increasing IOP (*i.e.*, very minimal facility), can make up the required total aqueous outflow (to equal total aqueous humor inflow), but only when IOP rises very rapidly to very high levels, not typically seen in POAG. In such cases, where IOP is extremely high, there is likely also a significant reduction in total aqueous humor inflow rate (Bill, 1971; Todd & Woodhouse, 1973). However, in POAG,

where IOP rises only moderately, there is no significant reduction in the total aqueous humor inflow rate (Martin, Fernández-Vila & Pérez, 1995).

An ability to measure $C_{(tot)}$ is therefore of importance when establishing, validating, or utilizing animal models of POAG. The mouse has been utilized for the study of this disease for over two decades at this point. The reasons for this are several-fold. Mice are relatively inexpensive and easy to house. Their relatively brief life span means that desired conditions can be developed rapidly, and they can be aged out within a reasonable time frame. There are more than 20,000 transgenic strains of mice now commercially available, and thus, many genes can be knocked in, knocked out, knocked up, or knocked down, thus facilitating studies on the disease at the genetic level. Further, there are many similarities between the mouse and human genomes. The anterior segment of the mouse eye has many features in common with the human eye in terms of structural anatomy, physiological function, innervation, pharmacological receptor expression, and blood supply. Also, the mouse TM and aqueous outflow pathway distal to the TM is structurally and functionally very similar to that of the human (Millar *et al.,* 2015; Lopez *et al.,* 2017), which sets mice apart from most other placental mammals, with the exception of non-human primates and rats. Until the advent of the 21st century, POAG studies on mice were limited as accurate and repeatable measurements of IOP in these animals were difficult to perform, and reliable measurements of aqueous humor dynamics (AHD) were thought to lie outside of the limits of available instrumentation due to the small size of the eyes of this species and the relatively tiny volumes of fluid involved. However, improvements in instrumentation have now led to the ability to perform both of these tasks in these animals routinely.

Here, we describe a protocol for the measurement of $C_{(tot)}$ in living mouse eyes using a technique of constant flow rate infusion of perfusate into the aqueous chamber of the eye (Millar, Clark & Pang, 2011; Millar *et al.,* 2015). For illustrative purposes, some representative data obtained using this technique is also presented. During infusion, pressure developed within the system is monitored *via* a pressure transducer. The apparatus may also be utilized to obtain a value for episcleral venous pressure (EVP). Using this data, it is then also possible to derive estimates for aqueous humor formation rate (F_{in}) and uveoscleral outflow (F_u) by substitution into the modified Goldmann equation (Goldmann, 1955; Gabelt & Kaufman, 2011), which specifies a good approximation of a value for IOP in terms of F_{in}, F_u, C_{tot}, and EVP:

$$IOP = \left[\frac{Fin - Fu}{Ctot}\right] + EVP$$

CHAPTER 35

Aqueous Humor Dynamic Measurements in Nonhuman Primates

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Abstract: This chapter describes the protocols to noninvasively measure the parameters of aqueous humor dynamics (AHD) in nonhuman primates (NHPs). These parameters define intraocular pressure (IOP) as described in the modified Goldmann equation, and changes to these parameters can alter IOP. Methods to access these parameters are used to study glaucoma and to examine new treatment ideas to lower IOP. The methods to access AHD include measurements of IOP by pneumatonometry, aqueous flow by fluorophotometry, and outflow facility by two methods, tonography and fluorophotometry. Uveoscleral outflow and episcleral venous pressure cannot be measured noninvasively in NHPs. Instead, episcleral venous pressure values in NHPs are obtained from the literature, and uveoscleral outflow is mathematically calculated using the modified Goldmann equation. These methods have been used in NHPs with ocular normotension, naturally occurring glaucoma, and experimental ocular hypertension. Preclinical studies of AHD are often conducted on NHPs before progressing to phase I clinical trials.

Keywords: Aqueous humor dynamics, Aqueous flow, Fluorophotometry, Intraocular pressure, Ocular hypertension, Outflow facility, Tonography, Tonometry, Uveoscleral outflow.

INTRODUCTION

The anatomy and physiology of nonhuman primates (NHPs) are similar to humans in many ways, making the NHP an important model in defining and understanding the pathogenesis of human disease. They respond to pharmacological drugs in a manner similar to humans and are often used to study

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novel treatments for human conditions. One important disease in humans that NHPs have helped to understand is glaucoma, a potentially blinding ocular disease of humans around the world. Elevated intraocular pressure (IOP) is the primary modifiable risk factor for glaucoma; lowering IOP by surgical or pharmacological means is its primary treatment.

Intraocular pressure is maintained by the fluid dynamics in the anterior segment of the eye. The key equation describing the factors involved in aqueous humor dynamics (AHD) and their relationships to each other is the Goldmann equation. It was developed over 70 years ago (Brubaker, 2004), and the modified Goldmann equation remains an important research tool today. It defines IOP as (Fa - Fu)/C + EVP. Fa (µl/min) is the rate of flow of aqueous humor from the posterior chamber, where it is secreted into the anterior chamber, where it circulates and drains. Fa is an estimate of the rate of aqueous humor production. C (µl/min/mmHg; the inverse of resistance) is the facility of outflow of aqueous humor from the eye. C is primarily, but not solely, located within the trabecular outflow pathway. Fu (µl/min) is uveoscleral outflow, the flow rate of aqueous humor around and through uveal tissues and sclera. EVP (mmHg) is the episcleral venous pressure, the pressure in the vessels that drain aqueous humor from the trabecular outflow pathway. Numerous methods, both invasive and noninvasive, have been developed to assess these parameters (Toris *et al*., 2021). Although the noninvasive methods tend to be less accurate than invasive methods, they cause no lasting effects and can be repeated, which is essential for studies with a longitudinal design.

The purpose of this report is to describe the noninvasive methods to measure parameters of AHD in nonhuman primates, primarily cynomolgus and rhesus macaques. These methods have been performed with the approval of the Institutional Animal Care and Use Committees at the institute where the primate research was done and in compliance with the Statement for the Use of Animals in Ophthalmic and Visual Research from the Association for Research in Vision and Ophthalmology (ARVO).

EXPERIMENTAL PROTOCOLS

Three of the parameters in the Goldmann Equation can be measured noninvasively in monkeys, preferably on the same day. IOP is measured by tonometry, Fa by fluorophotometry, and C by tonography and/or a fluorophotometric method. EVP cannot be measured noninvasively in the cynomolgus macaque because the vessels are not visible under the heavily pigmented conjunctiva of this species. Fu is not measurable noninvasively but is assessed mathematically by the rearranged Goldmann equation: Fu = Fa - C(IOP-

EVP). IOP, Fa, and C are the measured values, and EVP is taken from a paper by Maepea and Bill (Maepea and Bill, 1989). They measured EVP directly using microneedles and reported EVP at several different IOPs set experimentally. Fu is a highly variable term because it is calculated from parameters with their own inherent variability. Changes in Fu from an experimental manipulation may not be detectable unless the changes and/or sample size are large (Fig. **1**).

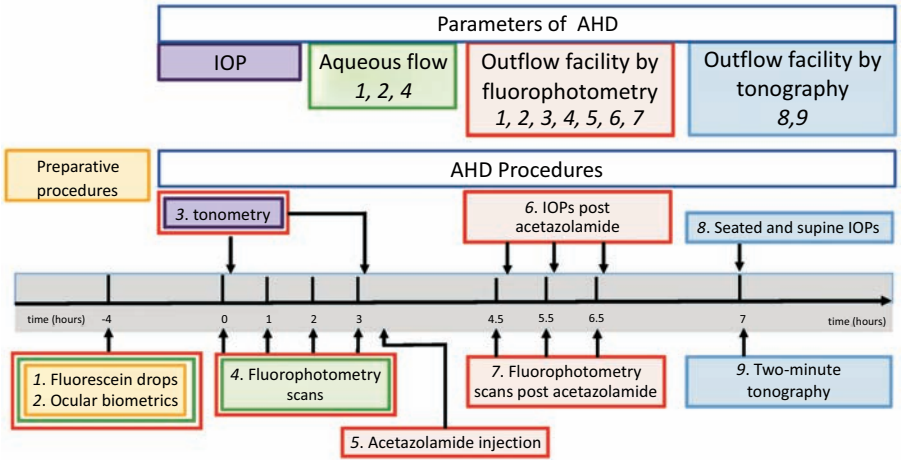

Fig. (1). Timeline (grey box) of study procedures to measure IOP (purple), aqueous flow (green), outflow facility by fluorophotometry (red), and outflow facility by tonography (blue). Numbers in the parameters boxes indicate the numbers of the procedures that are needed to make the parameter assessment. Color-coded outlines around procedure boxes indicate measurements needed for a particular procedure. Preparative procedures are in gold, and they can be performed 4 or more hours before the first fluorophotometry scan. AHD, aqueous humor dynamics; IOP, intraocular pressure.

Tonometry

The pneumatonometer, Tonolab, and Tonopen tonometers can be used in NHPs. These instruments are used in humans in accordance with the manufacturer's instructions.

a. Equipment: Our preference is the pneumatonometer for several reasons. The pressure readings can be saved in a digital format. Pressures are collected for multiple seconds to minutes and graphed. A section of the IOP graph can be deleted if there is excessive movement during data collection. This happens frequently with awake, curious animals. An IOP measurement of a supine sedated animal is shown in Fig. (**2**). Under anesthesia, there is not much of a pulse wave.
b. Supplies: Artificial tears, such as EyeStream (Alcon), proparacaine hydrochloride, 0.5% (Alcaine; Alcon, Ft. Worth, TX), report forms (one for each animal), pen, soft tissue wipes, and pen light.

CHAPTER 36

Protocols for Pre-Clinical Testing Microshunts to Drain AQH for Glaucoma

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Abstract: This chapter summarizes and describes most of the test methods used for pre-clinical testing of a permanently implantable glaucoma drainage device called the PRESERFLO® MicroShunt (InnFocus Inc./A Santen Co.) The tests are required by most medical device regulatory authorities as technical files or experimental data to demonstrate safety and subsequently to advance testing in humans to demonstrate efficacy and final approval for commercialization. There are many international standards and guidelines, such as the International Organization for Standardization (ISO), American Society for Testing and Materials (ASTM), United States Pharmacopeia (USP), and American National Standard Institute (ANSI), to name a few, which help in the harmonization of testing required to show the device is designed and functions as expected. Glaucoma drainage devices are typically shunts that divert aqueous humor from the anterior chamber to the exterior spaces of the eye by bypassing some of the common pathways, such as the trabecular meshwork or choroidal space. Some devices attempt to enhance the obstructed pathways and are made of metal, plastic, or degradable materials. The test methods described herein are compiled to educate the reader of pre-clinical tests required, with the understanding that each device model is unique, requiring customization of methods and/or tooling to accommodate the test requirements using the design at hand.

Keywords: Anterior chamber, Biocompatibility, Bond test, Compression test, Carcinogenicity test, Ethylene oxide residual test, Eye, Flow test, Exhaustive extraction test, Genotoxicity test, Glaucoma drainage device, Genotoxicity test, Intraocular pressure, Leachable test, Microshunt, Pre-clinical test, Protocol, Pyrogen test, Shelf-life test, Transportation test, Test method, Visual inspection.

INTRODUCTION

Pre-clinical testing of glaucoma drainage devices has been ongoing since 1976, when the first devices began to be regulated by the governing agencies, such as

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the Food and Drug Administration (FDA). Since then, there have been efforts to come up with guidance and recommendations that are recognized by regulatory bodies to harmonize and help companies to design safe implantable devices. One such organization in the medical ophthalmic field is the Z80 committee, accredited by the American National Standard Institute Inc. (ANSI), founded in 1956, and further vision council subcommittees formed in 2009. The original and first American Glaucoma device standard came about in 2001, and a revised version was released in 2014 as ANSI Standard Z80.27-2014, American National Standards for Ophthalmics-Implantable Glaucoma Devices.

This section will describe some general test methods or protocols that were developed to demonstrate the safety of the PRESERFLO MicroShunt. By no means do these protocols represent standard test methods; however, they were designed to demonstrate and satisfy the guidelines and recommendations at that time of development. It should be understood that new implantable glaucoma devices, whether designed as permanent devices or degradable devices, may have unique properties or characteristics where new test methods must be developed to satisfy the guidance or supplemented to show the device is safe to proceed first in human and clinical studies, thereof.

EXPERIMENTAL PROTOCOLS

Although these protocols will help guide, for example, the type of testing required for pre-clinical testing of glaucoma drainage devices, it must be understood that depending on the design of the device, test methods need to be developed by the developer, which may be necessary and unique to its design. For example, a device that may have electronic components or has been designed to be active rather than passive devices will likely need additional testing and test methods not described here. It is the responsibility of the developer to assess the pre-clinical tests necessary to show safety and efficacy before moving onto human clinical trials.

Physical and Mechanical Assessments

Visual Inspection

- Prepare a stereo microscope or microscope that has a magnification of 10X and obtain forceps and a clean Petri dish filled with a 1-2 mm depth of isopropyl alcohol. Also, prepare a separate dish for reject devices.
- Obtain the device to be inspected and immerse it in the isopropyl alcohol, allowing the lumen to be filled with fluid as opposed to air bubbles.

- Inspect the device from one end to the other at 10X. Refer to standard photos for acceptable and non-acceptable defects and sort accordingly. These defects can range from surface roughness to lumen defects and inclusions.
- Record the type of un-accepted defects for each of the rejected units.

Compression Test

- Set up the material tester machine (*e.g.*, Instron Machine) with a 50N load cell with a custom top and bottom compression tool held by the pneumatic or mechanical flat grips. Set an automatic stop to 10N to prevent accidental over-compression, which can damage the load cell. Set compression test speed at 700 μm/min.
- Manually, by jogging the machine, bring the top grip down until the top and bottom compression tools touch. Reset or zero that position on the machine, as this will be the reference position.
- Move the top grip up to leave space to place the device onto the bottom compression tool and place the device into position.
- Manually jog down the top grip until the top compression tool is just touching the device (usually the tube). This should be a position reading close to the outer diameter of the tube.
- Start the compression test until 50% of the tube is compressed or until ½ of the outer diameter is compressed. Record the load at 50% compression.

Gravimetric Flow Test

- Set up the gravimetric flow test apparatus, which consists of a weight balance, a water reservoir connected to a tube to a 3-way connector, and an adjustable platform for the water reservoir.
- The 3-way connector is mounted with a clamp holder at a fixed height, and the water reservoir is filled with clean water and checked to ensure there are no air bubbles throughout the tube and connector.
- The water reservoir height is adjusted on the platform to achieve a head pressure of 20 ± 1 mmHg using a calibrated manometer connected to the 3-way connector.
- The front end of the MicroShunt is mounted into a 21G 10 mm long needle and glued with silicone rubber adhesive to prevent a leak. This needle with a device is then primed with filtered water to be mounted onto the 3-way connector facing down.
- The needle with the device is carefully connected to the 3-way connector, avoiding any air bubbles. A small glass vial with water is placed on the balance. The 3-way connector mounted on the stand is placed inside the balance chamber such that the water drips into the vial. Add water in the vial such that the tip of

SPONTANEOUSLY/NATURALLY OCCURRING AND GENETICALLY ENGINEERED ANIMAL MODELS OF OCULAR HYPERTENSION (OHT)/GLAUCOMA

CHAPTER 37

Protocols for the Use of DBA/2J Mice for Pigmentary Glaucoma Research

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Abstract: Pigmentary glaucoma (PG) is a subtype of secondary open-angle glaucoma defined by iris melanin granules or pigment deposits in the trabecular meshwork. Morphological and functional changes in the aqueous humor outflow pathways lead to increased intraocular pressure and progressive damage to the optic nerve, which could lead to vision loss. The DBA/2J mouse has become an indispensable resource in scientific research, providing insights into the underlying pathophysiology of PG. In this chapter, we describe three methods pertinent to the study of PG utilizing the DBA/2J mouse model: intraocular pressure (IOP) measurement, pattern electroretinography (PERG) as a measurement of inner retinal functional assessment of vision, and a modified Fontana-Masson staining (FMS) technique for quantitative measurement of melanin pigments.

Keywords: Fontana-Masson stain, Pigmentary glaucoma, Pattern electroretinogram, Trabecular meshwork, Intraocular pressure.

INTRODUCTION

Pigmentary glaucoma (PG) is a type of secondary open-angle glaucoma characterized by the accumulation of pigment granules in the trabecular meshwork (TM), leading to increased intraocular pressure (IOP) and optic nerve damage. It typically affects males in their third to fifth decades of life (the male-female ratio is around 3:1) and is more prevalent in myopic patients (Niyadurupola and Broadway, 2008; Potash *et al*., 1994; Di Pippo *et al*., 2020).

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Pigment dispersion syndrome (PDS) and PG reflect different stages on a spectrum of the same condition. PDS occurs when anomalous iridozonular contact leads to rubbing between the posterior iris epithelium and lens structures (Liebmann *et al*., 1995; Okafor *et al*., 2017; Campbell, 1979). This rubbing leads to the liberation and dispersion of pigment particles that accumulate in the TM, causing TM endothelial cell necrosis (Niyadurupola and Broadway, 2008). The clinical presentation of PDS is defined by the triad of dense pigmentation of the TM, pigment localized on the corneal endothelium called Krukenberg's spindle and radial mid-periphery iris transillumination defect (Liebmann *et al*., 1995; Okafor *et al*., 2017). PG is defined when these characteristics cause an increase in IOP and glaucomatous optic neuropathy (Fig. **1**) (Scuderi *et al*., 2019; Okafor *et al*., 2017). Not all cases of PDS progress to develop PG. The risk of developing PG as a result of PDS has been estimated to be between 35-50% (Okafor *et al*., 2017).

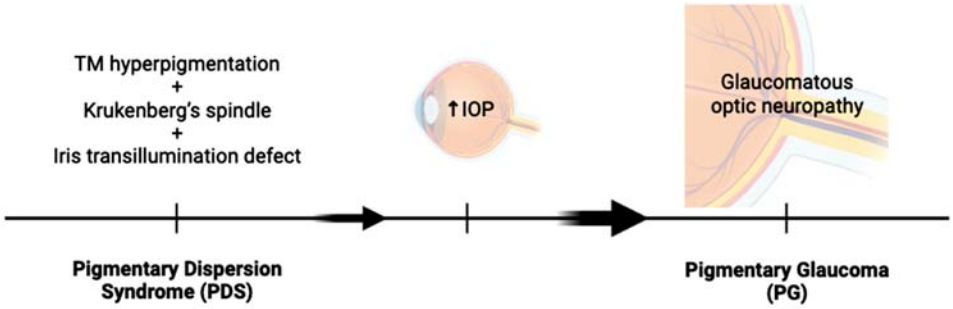

Fig. (1). Pigment dispersion syndrome and pigmentary glaucoma constitute stages of a spectrum of the same disease. PDS is characterized by the triad of TM hyperpigmentation, Krukenberg's spindle, and iris transillumination defect. PG is defined when PDS characteristics progress to cause glaucomatous optic neuropathy.

When diagnosing PG, the standard workup procedures for glaucoma are performed, including fundoscopy, optical coherence tomography (OCT), slit-lamp examination, and gonioscopy. In cases of PG, fundoscopy reveals glaucomatous optic nerve neuropathy, OCT shows retinal nerve fiber layer (RNFL) thinning, slit-lamp examination exhibits the presence of Krukenberg's spindle and iris transillumination defects, and gonioscopy displays homogenous pigmentation on the TM (Scuderi *et al*., 2019). In addition to these standard procedures, ultrasound biomicroscopy (UBM) and anterior segment OCT (AS-OCT) are performed to confirm the presence of iridozonular contact (Scuderi *et al*., 2019; Okafor *et al*., 2017; Potash *et al*., 1994).

The DBA/2J mouse strain (John *et al*., 1997; John *et al*., 1998) is a widely used animal model for studying PDS and PG. Mutations in the *Tyrp1* and *Gpnmb* genes, which code for melanosomal proteins, contribute to iris atrophy and

pigment dispersion in DBA/2J mice (Wang and Dong, 2016; Anderson *et al*., 2002). As a result, DBA/2J mice develop an age-related form of glaucoma that displays the clinical features of PDS and PG, with most of these mice beginning to develop iris pigmentation abnormalities or dispersion by six months and optic nerve damage by eight months (Lu *et al*., 2011; Pu *et al*., 2022).

There are a variety of experimental techniques that can be used to study the pathogenesis of PG in DBA/2J mice. These include pharmacological interventions, such as topical or systemic administration of drugs to lower intraocular pressure, and genetic manipulations, such as using CRISPR-Cas9 to knock out or knock in specific genes. Additionally, histological techniques, such as immunohistochemistry and electron microscopy, can be used to study the accumulation of pigments in the trabecular meshwork and the subsequent changes in the structure and function of the meshwork. In this chapter, we describe three protocols relevant to studying PG in DBA/2J mice: IOP measurement, pattern electroretinography (PERG), and a modified Fontana-Masson staining (FMS) technique.

INTRAOCULAR PRESSURE MEASUREMENT

Measuring IOP in DBA/2J mice is an essential step in assessing the development and progression of PG. Several methods can be used to measure IOP in mice, including invasive and non-invasive methods.

Invasive methods for measuring IOP in mice include cannulation of the anterior chamber and micro-tip manometry. Cannulation of the anterior chamber involves inserting a small tube through the cornea and into the anterior chamber and measuring the pressure directly. Micro-tip manometry involves inserting a small probe into the eye through a small incision and measuring the pressure directly. These methods are more accurate than non-invasive methods but require anesthesia and have a higher risk of complications such as opacity and perforation (Morris *et al*., 2006; Danias *et al*., 2003).

One of the most commonly used non-invasive methods for measuring IOP in mice is the Tono-Pen, which uses a small probe to measure the resistance to indentation of the cornea. Another non-invasive method is the rebound tonometer, which measures the rebound of the cornea after a small impact. Both of these methods are quick, easy to perform, and have a lower risk than invasive methods (Morris *et al*., 2006). Anesthesia can be used, but it is important to consider the potential effects of anesthesia on IOP measurements, as certain anesthetics can lower IOP (Danias *et al*., 2003). In the following section, we describe the steps to measure IOP using a TonoLab tonometer, which has been found to accurately measure IOP in normal and DBA/2J mice (Pease *et al*., 2011).

CHAPTER 38

Protocols for Creating Genetically-Transduced Animal Models of Ocular Hypertension and Glaucoma

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Abstract: Several animal models of ocular hypertension (OHT) have been developed over the last two decades. Some researchers simply use naturally available naïve mammals such as rodents, rabbits, dogs, and monkeys to evaluate the effects of test agents. Other OHT researchers have created rodent models by ablating episcleral veins on the corneal surface using lasers or clips or filling them with hypertonic saline. In monkeys, the trabecular meshwork, the major aqueous humor (AQH) drainage system from the anterior chamber of the eye, can be partially coagulated using a laser to increase intraocular pressure (IOP). With the advent of genetical techniques, scientists have created gene knockout models of OHT. In the current chapter, the authors describe methods to genetically deliver genes into the anterior chamber and/or specific tissues that transduce the production of fibrotic cytokines such a transforming growth factor-beta (TGF-β_2). This cytokine is abnormally elevated in AQH of OHT/glaucoma patients and is increasingly linked to the development of OHT/glaucoma.

Keywords: Genes, Glaucoma, Ocular diseases, Ocular hypertension, Viral vectors.

INTRODUCTION

Ocular hypertension (OHT) or elevated intraocular pressure (IOP) is one of the major culprits implicated in the pathogenesis of many forms of glaucoma, an insidious, asymptomatic silent thief of eyesight (Weinreb *et al*., 2014; Sharif, 2017, 2021). It is well known that the inflammatory and fibrosis-causing cytokine, transforming growth factor-beta (TGF-β2), is naturally elevated in the aqueous humor (AQH) of patients with OHT/glaucoma (Trapathi *et al*., 1994; Yamamoto

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et al., 2005) and is responsible for inducing the production and accumulation of collagen and fibronectin in and around the trabecular meshwork (TM) (Fuchshofer *et al*., 2005; Shephard *et al*., 2010). The clogging of the TM causes the subsequent excess accumulation of AQH in the anterior chamber of the eye, and this results in an increase in IOP (Weinreb *et al*., 2014; Sharif, 2017, 2021). The resultant OHT over several decades damages the optic nerve and kills the retinal ganglion cells, which transmit electrochemical signals to the brain for decoding and visual perception. Chronic OHT leads to loss of vision and can lead to blindness if not diagnosed and treated in a timely manner (Weinreb *et al*., 2014; Sharif, 2017, 2021).

In an effort to better understand the molecular and cellular mediators of OHT and how this affects the visual apparatus and the loss of vision, researchers have created many types of animal models of elevated IOP/OHT and many are described in this book. The topic of the current chapter is the way genetic techniques have been deployed to recapitulate a human version of OHT in a rodent model by applying the known involvement of TGF-beta in the process of IOP elevation. Such genetic manipulation has been successfully rendered in mice and rats and will be described below using adenoviral delivery (Shephard *et al*., 2010) and lentiviral delivery methods (Patil *et al*., 2022).

ANIMAL HANDLING

All animals should be treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO), and the animal handling and testing protocols must be approved by the institutional Animal Care and Use Committee. It is recommended that Wistar rats (2– 4 months old) and BALB/cJ mice (2–5 months old) were acquired from Charles River Labs (Wilmington, MA) and Jackson Labs (Bar Harbor, ME). The rodents should be adequately housed, maintained on a 12/12 hours light/dark cycle, and offered food and water ad libitum.

VIRAL VECTOR PRODUCTION AND DELIVERY OF TGF-B2 TO ELEVATE IOP

1. Obtain wild-type (WT) human transforming growth factor β2 (hTGFβ2) cDNA (NM_003238) in the pCMV6-XL5 vector from a reputable source.
2. Next, generate the cDNA coding for the active form of hTGFβ2 by converting the cysteines at positions 226 and 228 to serines.
3. Synthesize the region extending from the HindIII site (nt 1976) to the PpuMI site (nt 2395) so that nucleotides 1985 and 1991 are changed from guanine to cytosine, converting the cysteines to serines at amino acids 226 and 228 of the hTGFβ2 protein as mentioned above.

4. Next, clone this fragment into pCMV6- XL5.hTGFβ2 and then subclone the wild-type and mutant coding region cDNAs into the pacAd5.CMV.KN.pA shuttle vector using EcoRI/Xba1 restriction.
5. Use CsCl gradient centrifugation to purify the viruses, and then dialyze against Tris-EDTA buffer containing 10% sucrose and store at -80°C. Test each virus for wild-type revertants and titer by polymerase chain reaction and A549 plaque assay. In all cases, use empty adeno-associated virus (AAV) (Ad.Empty) as a negative control.
6. Confirm that the animals to be used are free of any apparent ocular disorders by direct ophthalmoscopic examination.
7. Anesthetize rats *via* an intramuscular injection using a solution of acepromazine (3 mg/kg), ketamine (33 mg/kg), and xylazine (7 mg/kg). For mice, perform a single intraperitoneal injection of a solution containing acepromazine (1.8 mg/kg), ketamine (73 mg/kg), and xylazine (1.8 mg/kg;).
8. When performing intracameral injections, instill 1-2 drops of 1% cyclopentolate (Mydriacyl) to dilate the pupil. Topical ocularly instill 1-2 drops of 0.5% tropicamide (Alcaine) to locally anesthetize the ocular surface of the eye.
9. Then perform intravitreal or intracameral injection of a suspension of adenovirus of a specified titer in a volume of 5 μL for the rat eye and 2 μL for the mouse eye using a Hamilton glass microsyringe fitted with a custom-made 1-inch 33-gauge needle with a 10° bevel. The uninjected contralateral eye should be used as a control. The injections should be performed slowly over 30 seconds. Leave the needle in place after the injection is completed for a minute to prevent fluid reflux and then withdraw slowly from the eye. Allow the rats to recover from all the aforementioned procedures.
10. For taking the IOP measurements, ensure the investigator is masked to the treatment paradigm. Use a rebound tonometer (TonoLab; Tiolat Oy; Helsinki, Finland) to take several measurements for each eye at each time point (before and after the injections).
11. A pictogram of the procedure for the lentivirus-delivered TGFβ2 gene and its expression *in vivo* in mice is shown in Fig. (**1**). Furthermore, the elevation of IOP by this genetic means is shown in Fig. (**2**).

RNA Isolation from Rodent Tissues

1. Euthanize the animals at various times following AAV vector-genetic construct injection using the CO_2 asphyxiation method, enucleate the eyes, and dissect them into the anterior and posterior segments.
2. Place each segment in an individual microfuge tube that contains 1 mL Trizol reagent, 1 μL GlycoBlue (glycogen covalently coupled to blue dye), and one 5-

CHAPTER 39

Protocol for Conditional Knockout Mouse Model of OHT and Glaucoma

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Abstract: This chapter describes the protocols used to create a conditional knockout mouse model to study partially closed-angle glaucoma where the intraocular pressure (IOP) is raised beyond normal levels. The Cre/loxP approach was employed as a strategy to conditionally inactivate the AP-2β gene from the developing periocular mesenchyme (POM) that is responsible for creating the trabecular meshwork (TM) (Taiyab *et al*., 2022). The Mgp-Cre knock-in (Mgp-Cre.KI) mice, in combination with the AP-2β floxed mice, were utilized to create a targeted deletion of AP-2β to the TM area. The AP-2β TMR KO mutants exhibit an absent TM and underdeveloped Schlemm's canal (SC) and partial adherence of the iris to the cornea. The mutants have significantly higher IOP than their wild-type littermates by one month of age, and this is correlated with a progressive, significant loss of retinal ganglion cells, reduced retinal thickness, and reduced retinal function, as measured by electroretinography. Thus, these mutant mice can serve as a model for understanding and treating progressive human primary angle-closure glaucoma with associated ocular hypertension.

Keywords: Anterior segment dysgenesis, Conditional KO, Eye, Glaucoma, Intraocular pressure, Ocular hypertension, Optic nerve, Protocol, Retina.

INTRODUCTION

Appropriate development of structures in the anterior segment of the eye is critical for normal ocular functioning. Proper development of the aqueous humor outflow structures, including the trabecular meshwork (TM) and Schlemm's canal (SC), is necessary to ensure that there is a balance between aqueous humor (AQH) production and drainage, thus maintaining optimal intraocular pressure (IOP). Glaucoma due to ocular hypertension (OHT) can occur due to an imbalance of aqueous humor (AQH) generation and its egress, which can result in the building

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of IOP. This ultimately causes pressure and damages the optic nerve head (Dietze *et al*., 2022). While there are many types of glaucoma, primary open-angle glaucoma and angle closure glaucoma are two major forms (Taiyab *et al*., 2022). In primary open-angle glaucoma (POAG), there is a build-up of aqueous humor fluid in the anterior chamber due to an increased resistance to AQH flow through the trabecular meshwork (TM) and Schlemm's canal (SC) drainage structures responsible for conventional outflow. However, in primary angle closure glaucoma (PACG), these structures are completely blocked, causing the conventional pathway to become inaccessible. One of the underlying conditions that can cause angle closure glaucoma is referred to as peripheral anterior synechia (PAS), a condition in which the iris adheres to the cornea (Lee *et al*., 2006). Additionally, aberrations in the development of the anterior structures (cornea, iris, lens, the cornea, ciliary body, trabecular meshwork, and Schlemm's canal) can lead to anterior segment dysgenesis (ASD) and disorders, and individuals with ASD are more susceptible to glaucoma (Taiyab *et al*., 2022). Importantly, glaucoma is one of the leading causes of blindness in the world, with an estimated 76 million people affected by it, and this number is projected to increase by almost 30% over the next decade (Quigley & Broman, 2006).

Current treatments for primary angle closure glaucoma include topical treatments such as prostaglandin analogs that lower IOP by increasing outflow or aqueous suppressants that help reduce the production of aqueous humor. Although several medications assist in managing PACG during its early stages, severe conditions require laser or surgery to expand the drainage angle or create an opening in the iris to allow for outflow (Wright *et al*., 2016).

Investigating the genetic pathways and mechanisms that govern the proper development of the tissues within the anterior segment of the eye will lead to a better understanding of the underlying cause of ASD and angle closure glaucoma. Anterior segment tissue development in mice is regulated by a collection of genes (*e.g.*, *Pitx2, Foxc1, Foxc2, Lmx1b, and Pax6)* (Chen & Gage, 2016; Cross *et al*., 2014; Liu & Johnson, 2010; Romero *et al*., 2011; Smith *et al*., 2000; Tümer & Bach-Holm, 2009) and the activating protein 2 (AP-2) family of transcription factors (*e.g.*, AP-2α and AP-2β) (Barzago *et al*., 2017; Bassett *et al*., 2007, 2010, 2012; Chen & Gage, 2016; Kerr *et al*., 2014; Pontoriero *et al*., 2008; West-Mays *et al*., 1999). AP-2α and AP-2β encoded by Tfap2a and Tfap2b genes, respectively, are both highly expressed in periocular mesenchyme (POM)-derived structures at earlier stages of embryogenesis, but only *Tfap2b* is specifically expressed within POM cells during the secondary migration phase (Bassett *et al*., 2012)(West-Mays *et al*., 1999). Importantly, we have demonstrated that the AP-2β family member, encoded by the *Tfap2b* gene, is present within POM cells that move to the area between the cornea and the anterior rim of the optic cup and

subsequently develop into the TM and SC (Bassett *et al*., 2012; Martino *et al*., 2016). To ascertain the role of AP-2β in the creation of the latter, we recently used the Cre/loxP approach to conditionally delete AP-2β from the evolving POM. This conditional deletion enabled targeting of the TM area and avoided the perinatal death observed in the AP-2β germline KO mutants.

The Cre/loxP approach is a refined strategy for conditional gene inactivation that relies on the DNA recombinase Cre and its recognition (loxP) sites. For conditional mutagenesis, a target gene is modified by the insertion of two loxP sites that enable to excise the flanked (floxed) gene segment through Cre-mediated recombination. Conditional mutant mice are obtained by crossing the floxed strain with a Cre transgenic line such that the target gene becomes inactivated *in vivo* within the expression domain of Cre. Our laboratory employed this approach using the MgpCre transgenic mouse system (Borrás *et al*., 2015) combined AP-2β floxed mice (Martino *et al*., 2016; Van Otterloo *et al*., 2022) to target AP-2β from the POM cell population that creates the TM. These mice have been termed the AP-2β-TM-region knockout mice (AP-2β TMR-KO). Previous work has shown that the expression of MgpCre is confined to the iridocorneal angle and the peripapillary scleral region (Borras *et al*., 2015). Our lab was the first to demonstrate that the expression of MgpCre occurs earlier in the eye during embryonic development at E15 and, importantly, in the POM (Taiyab *et al*., 2022). This new knowledge inspired us to create a conditional KO model of AP-2β in the developing TM region using the MgpCre (Fig. **1**).

Deletion of AP-2β using the Mgp-Cre knock-in (Mgp-Cre.KI) from the developing TM area resulted in offspring with an absent TM and underdeveloped SC as ascertained by the reduction in key markers (*e.g.*, αSMA, myocilin, Prox1, and endomucin) (Taiyab *et al*., 2022). There was a partial adherence of the iris to the cornea with the iridocorneal angle partially closed, and this resulted in significantly elevated IOP by 1-month of age. Moreover, elevated IOP correlated with decreased retinal thickness, progressive loss of RGCs, and thus decreased ERG responses. The elevated IOP was temporarily reduced following latanoprost treatment, thus indicating the functionality of the uveoscleral pathway in this model (Taiyab *et al*., 2022).

Although there are a variety of mouse models that have been developed to understand the progression of glaucoma (*e.g.*, optic nerve crush model, microbead injection model, and DBA/2J model) (Johnson & Tomarev, 2010)), there are fewer models where genetic mutation/deletion has been applied. Some of these models include mice with mutations in *Sh3pxd2b* (Mao *et al*., 2011)), *Pitx2* (Chen & Gage, 2016), *Foxc1*, *Foxc2* (Smith *et al*., 2000), and *Lmx1b* (Liu & Johnson, 2010), as well as AP-2β NCC KO mice (Martino *et al*., 2016). All these models

DRUG FORMULATION, PHARMACOKINETICS, AND TOXICOLOGY STUDIES FOR COMPOUNDS

CHAPTER 40

Protocols to Study the Drug Formulation and Stability Aspects of Anti-Ocular Hypertension and Anti-Glaucoma Drugs

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Abstract: In order to dissolve and make dilutions of test compounds for topical ocular delivery, a suitable formulation needs to be created that will deliver the molecule without causing ocular discomfort. Additionally, it is important to ensure that the formulation will not cause inflammation or induce serious ocular or systemic side effects. The physiochemical properties of the compound dictate the choice of the formulation that, in turn, will endeavor to control the pH, stability (solution, thermal, photo), and the overall characteristics of the drug in solution or suspension. Furthermore, the route of delivery of the test agent will also influence the choice of the formulation. Excipients may be added to enhance the ocular retention time and thus enhance the penetration of the drug across the cornea/conjunctiva in order to allow the active moiety to reach the aqueous humor in the anterior chamber of the eye.

Keywords: Drug delivery systems, Eye drops, Eye anatomy and physiology, Formulations, Glaucoma research, Inserts, ICH, Nanosuspension, Ocular barrier, Solution, Suspension, Stability, Topical delivery.

INTRODUCTION

This section describes a protocol to study the drug formulation and stability aspects of glaucoma research on the human eye. First of all, in order to develop an effective formulation for glaucoma research, it is important to understand the anatomy and physiology of the eye. The anterior segment of the eye includes the aqueous humor (AQH), cornea, iris, pupil, ciliary body, conjunctiva, and lens. However, the posterior segment contains the sclera, choroid, and retina, with the vitreous cavity containing the vitreous humor.

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Most anti-glaucoma drug formulations are developed as solution or suspension eye drops to instill on the anterior segment of the eye, the cornea. Anatomical (*e.g.*, conjunctiva, cornea) and physiological (*e.g.*, choroidal blood flow, efflux transporters) barriers limit the bioavailability and, thus, the efficacy of these conventional drug forms (Agrahari *et al*., 2017; Barar *et al*., 2016; Gaudana *et al*., 2010; Janagam *et al*., 2017). The most potent glaucoma drugs are BCS class 2 drugs (possess low aqueous solubility and high permeability) or BCS class IV drugs (possess low aqueous solubility and low permeability). The latter features of the drugs limit the efficacy of the topical ocularly delivered compounds. Also, an ideal ocular formulation should be easily self-administered to the eye, not cause ocular irritation, and garner patient acceptance/compliance. For this reason, eye drop solutions or suspensions are provided in easy-to-squeeze low-density polyethylene (LDPE) bottles with LDPE round or oval tips and polypropylene (PP) plastic caps, which are easy to remove for self-administration.

The bioavailability of topical formulations of drugs is adversely affected by many factors, such as solution drainage, blinking, tear turnover, and corneal/conjunctival tissue barriers. Consequently, perhaps only 1-7% of topical ocularly instilled drugs enter the AQH (Janagam *et al*., 2017; Ghate and Edelhauser, 2006). Moreover, tear drainage facilitates the removal of the drug from the eye. Consequently, ~ 5% of the instilled eye drop drug actually comes in contact with tissues within the anterior chamber of the eye to evoke the desired intraocular pressure lowering (Gaudana *et al*., 2010; Ali *et al*., 2016). Hence, new drug dissolution and delivery methods (*e.g.*, liposomes, nanoparticles, nanomicelles, nanosuspensions, dendrimers, implants, microneedles, and *in situ* thermo-sensitive gels) need to be developed beyond the existing suspensions, emulsions, ointments, and aqueous gels to treat ocular diseases impacting the front and the back of the eye (Barar *et al*., 2016).

To deliver drugs to the posterior eye segment, a variety of routes of administration have been developed, such as suprachoroidal and intravitreal, periocular (subconjunctival, sub-Tenon, and retrobulbar), and topical and systemic (Agrahari *et al*., 2017, Peptu *et al*., 2015). Topical ocular delivery is the least invasive and thus is the most used method, but the drawback is minimal delivery to the back of the eye (Barar *et al*., 2016). Not surprisingly, the most efficient and effective way for drugs to reach the retinal and optic nerve head tissues is when they are intravitreally delivered (Peptu *et al*., 2015).

Drug Formulations for Anti-Glaucoma Drug Research

There are many factors to consider when generating a good target product profile (TPP) for a drug to be formulated for ophthalmic application. The TPP attributes

under such consideration should include as many of the following as possible: patient population, intended target tissue, single or multi-dose medication, duration of therapy, ease of manufacturing and scalability, formulation, route of delivery, sterility, shelf-life, storage, pH, endotoxin levels, addition or omission of preservatives, dosing frequency, safety and efficacy, and package type (*e.g.*, bottle size, fill volume, and types of plug).

Generally, early formulations are either solutions or suspensions of eye drops. For solution formulation, drug concentration, solubility, pH, osmolality, solubility, sterility, preservative efficacy (PET), and stability (physical and chemical) are very important. For suspension formulation, appearance, pH, osmolality, viscosity, resuspension time, and settling are important parameters in addition to sterility, PET, and stability. As mentioned above, eyedrops are the easiest to formulate for topical ocular administration. However, limited efficacy is often the result of low bioavailability for drugs that need to penetrate the cornea/conjunctiva to reach their intended target tissue(s) (Jumelle *et al*., 2020; Souto *et al*., 2019). This is where the use of suitable excipients such as polyalcohol, hydroxymethyl cellulose, sodium carboxymethyl cellulose, or a combination of the latter can be used to enhance the ocular surface retention times and thus aid in an overall greater penetration to the anterior chamber (Patel *et al*., 2013; Achouri *et al*., 2013). Drug permeation can also be enhanced using certain preservatives (benzalkonium chloride), chelating compounds (*e.g*., EDTA), and mild detergents/surfactants (*e.g*., polyethylene glycol; bile salts). However, many studies have shown these agents to cause ocular surface irritation (Achouri *et al*., 2013).

Drug Substances (Active Pharmaceutical Ingredient)

Appropriate dose strength is critical due to poor bioavailability in ocular tissues, limitations and dosing volume, and ocular residence time. The physicochemical properties that are most common for the drug substance include molecular weight, logP, pKa, pH-solubility profile, solubility in co-solvent systems, solubility at various pH, chemical stability, degradation kinetics, and permeability. Key considerations for suspension formulations include crystallinity, particle size, melting point, hygroscopicity, surface area, polymorphism, density, non-homogeneity, aggregation and settling, and cake formation. Since the un-ionized form of the drug determines bioavailability, the ionization constant of the compound is an important determinant to consider. Hence, to obtain maximal un-ionization of the target compound at a pH close to 7.4, functional group selection present on the compounds is an important factor. Ultimately, such parameters should not impact the desired drug solubility, stability, and *in vitro* and *in vivo* potency. Additionally, knowledge and experience in improving the interfacial

CHAPTER 41

Protocol for an Ocular Pharmacokinetic Study in Rabbits for an Ocular Anti-hypertensive Drug

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Abstract: Ocular pharmacokinetics (PK) is an essential component of drug research and development, leading to the understanding of drug or drug candidate absorption, distribution, and elimination firstly in tissues targeted for efficacy and secondly in tissues/fluids that may be relevant to off-target effects (*e.g.*, toxicity). Rabbits are one species of choice for ocular PK due to greater eye size relative to rodents and lower expense relative to dogs or monkeys, as well as the wealth of published ocular PK data (and use in regulatory submissions). This chapter describes a generic protocol for assessing ocular PK for glaucoma and anterior eye segment research. The collection procedure is detailed but will vary depending on the objective of the PK study or the lab performing the study. As such, this is one, albeit relatively common, approach one can consider for studying ocular PK in rabbits.

Keywords: Drug distribution, Eye, Glaucoma, Ocular tissue, Ocular, Pharmacodynamics, PK/PD, Pharmacokinetics, Rabbit.

INTRODUCTION

Pharmacokinetic (PK) and pharmacokinetic/pharmacodynamic (PK/PD) studies have become essential for confirming drug exposure in target tissues and the relationship between drug exposure and efficacy. These assist the translational process of defining an efficacious dose in humans based on animal data. In the case of glaucoma research, ocular PK can be a stand-alone study or as part of a PK/PD study in an appropriate animal model. For compounds targeting the anterior eye segment (*e.g.*, control of intraocular pressure), one target tissue likely includes an iris-ciliary body (ICB or specifically the ciliary body). ICB is therefore collected and analyzed, along with the cornea, which is assessed for drug exposure at this ocular barrier, and aqueous humor from the anterior chamber, essentially representing a central compartment within the eye. Drug

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levels in the lens may be useful to assess accumulation within the lens for potential toxicity effects. Assessment of drug distribution to the posterior segment (behind the lens) can be useful to understand potential exposure to the retina (which can, at times, be collected as well). For reference, rabbit ocular anatomy is illustrated in Fig. (**I**). Various reviews of ocular PK and drug delivery, including application to glaucoma research and beyond, are available (Agrahari *et al*., 2016; Gaudana R *et al*., 2010; Urtti, 2006).

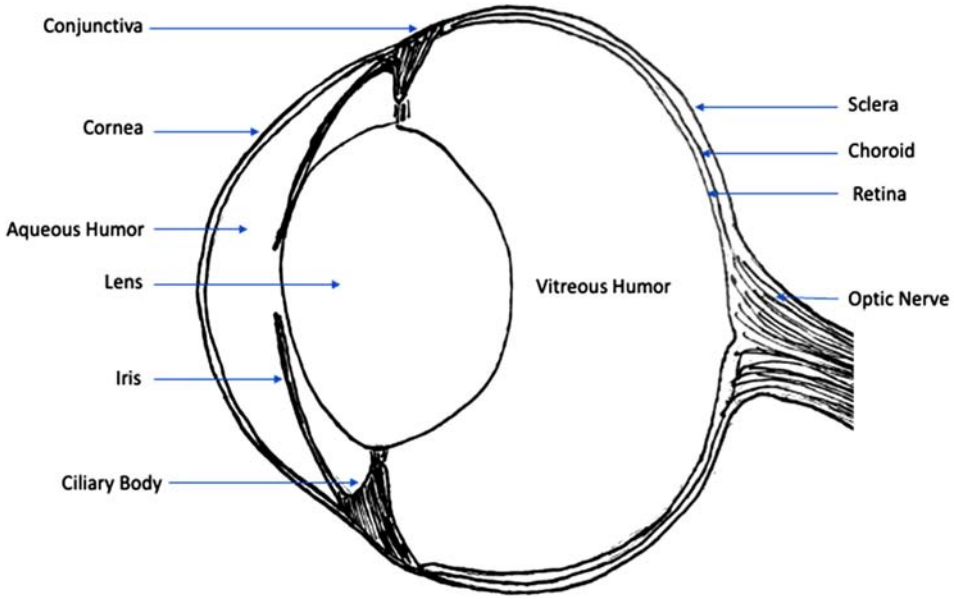

Fig. (1). Rabbit ocular anatomy.

The following describes a generic protocol for assessing ocular PK for glaucoma and anterior segment research. The collection procedure is detailed but can vary depending on the ultimate goal of the PK study or the particular requirement of the lab performing the study. Examples of ocular PK studies are those by Fayyaz *et al.* (2021), assessing the ocular PK of a beta-blocker cocktail, Shen *et al.* (2017), studying the ocular PK of topical bimatoprost, and Savinainen *et al.* (2019), studying the PK and intraocular pressure lowering activity of a drug candidate (PK/PD based on aqueous humor levels).

EXPERIMENTAL PROTOCOLS

The following procedures are performed for PK at predetermined time points following drug administration within a stand-alone ocular PK study or as part of a PK/PD study.

Materials

Isotonic saline (0.9%), gauze, 1cc tuberculin syringes with and without affixed needle (*e.g.*, 27G), weigh boat/paper, dissecting microscope (as needed), and pre-weighed (or tared) cryogenic polypropylene sample tubes with screw-caps (*e.g.*, 1.5mL and 15mL) are used. In addition, a weighing balance with at least 5-place precision with the goal of providing at least three significant figures for tissue weights is used. If blood is collected, a centrifuge for the separation of plasma fraction is needed.

Instruments

Tissue forceps with teeth, slightly curved microdissection forceps with teeth, slightly curved serrated microdissection forceps, curved corneal scissors (blunt tips), curved iris scissors (sharp tips), curved blunt scissors (enucleation scissors), eye scissors (straight or slightly curved with pointed tips), disposable scalpel with blade and Gill knife. These may be substituted as needed with similar instruments as required.

Animals and Treatment

Rabbits are commonly used due to the larger size of their eyes compared to rats and substantial published literature on ocular PK available for rabbits. This is despite differences between rabbit and human eyes, *e.g.*, the rabbit eye is slightly half the size of the human eye, substantially slower blink rate in rabbits, presence of a nictitating membrane, and only one punctum in the rabbit. Rabbits for PK can be non-pigmented (*e.g.*, New Zealand White) or pigmented (*e.g.*, Dutch-belted), depending on the degree to which the compound/drug is known or suspected to bind to melanin (pigment).

Investigative compounds are formulated as solutions or suspensions, preferably already tested for ocular compatibility and tolerance, and are administered by eye drop using a dropper (*e.g.*, droptainer) or a positive displacement pipet. The latter better ensures accurate volume, which is typically administered in the range of 35-40 microliters. Dosing is bilateral, *i.e.*, OS (left) and OD (right) eyes, except in the case where an undosed fellow eye is required for comparison, *e.g.*, in the case of safety assessment or to determine the contribution from systemic drug levels to ocular tissue levels *via* redistribution. Although drugs can be distributed from systemic circulation to the eye, this is less relevant for anterior segment tissues, for which the majority of drugs derive from local distribution from the ocular surface tissues, *e.g.*, cornea and conjunctiva. For the indication of glaucoma and IOP reduction, generally the anterior segment tissues are considered the drug target and are of primary interest for PK. As such, dosing is typically bilateral,

CHAPTER 42

Considerations for Ocular Toxicologic Pathology in Nonclinical Studies for the Development of Intraocular Biologics to Treat Glaucoma and Secondary Neurodegeneration

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Abstract: Ocular hypertension (OHT) associated with many forms of glaucoma can be treated by traditional pharmaceutical drugs, surgery, and aqueous humor microshunt implants. More recently, however, attempts have been made to introduce gene and cell therapies and even biologics to treat OHT and IOP-independent glaucomatous optic neuropathies with various degrees of success. In order to ensure the safety of any test agent or product, it is imperative that a set of toxicological studies be conducted on the compound or test agent. Accordingly, in this section/chapter, we provide considerations for toxicologic pathology evaluation of intraocular biologic therapeutic candidates to treat glaucoma. These types of investigations can be applied not only to biologics but, with appropriate modifications, to any entity that is to be introduced into animals and perhaps in humans in due course as part of proof-of-concept studies.

Keywords: Anterior and posterior segments, Drug development, Glaucoma, Neuroprotection, Retina, Toxicology studies.

INTRODUCTION

Considerations for identifying and selecting the clinical therapeutic candidate [utilizing various *in vitro* approaches and establishing *in vivo* proof of concept (diseased animals, KO, or normal animals)] are described in this book/volume elsewhere. The purpose of this section/chapter is to provide points for consideration to characterize the local (ocular) and systemic effects of intraocular administered biologic therapeutic candidates and to enable first-in-human clinical

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trials by executing a comprehensive toxicological program. Rather than being prescriptive and providing protocol-like settings, the goal of this chapter is to provide sufficient building blocks and considerations that can be adapted on a case-by-case basis for intraocularly (intracameral, IC or intravitreal, IVT) administered therapeutic biologic candidates/treatment modalities in an effort to treat glaucoma and provide neuroprotection.

The toxicology program is commonly performed in healthy animals (i.e., not a disease model) where only the potential ocular and systemic effects of the candidate therapeutic are characterized in the absence of other potentially confounding variables. The overall goal of the toxicological program is to characterize potential safety ocular and systemic safety risks for patients using nonclinical studies to build the weight of evidence, enable the clinical development plan (CDP), and initially enable first-in-human clinical trials. Thus, the CDP provides the foundation for toxicological program considerations in terms of dosing frequency and duration. This chapter will cover species selection, dose selection, study duration, dosing frequency, clinical ophthalmic and general toxicology endpoints, histopathology assessment, and integrated assessment of the totality of the toxicology data to interpret the data. In the absence of ocular-specific regulatory guidelines for intraocular biologic administration, guiding principles of ICH S6(R1) (preclinical safety testing requirements for biotechnological products) and ICH M3 (nonclinical safety studies recommended to support human clinical trials of a given scope and duration as well as marketing authorization for pharmaceuticals) will be applied as needed.

Species Selection

The nonclinical toxicology species selection is guided by pharmacological/pharmacodynamic assessment of the therapeutic candidate and includes (1) the evaluation of sequence homology, (2) binding, and (3) pharmacodynamic activity data for human vs. multiple laboratory species (*e.g.*, non-human primates (NHPs); minipig, dog, rabbit, rodent vs. human). Anatomical considerations include factors such as the size of the eye and the feasibility of intraocular dosing (intracameral, IC or intravitreal, IVT). These combined pharmacological and anatomic data support toxicology species selection to derive a relevant and accurate risk assessment for patients and establish a path forward. For intraocular administered biologics, NHPs are often the only pharmacologically relevant species. If the therapeutic candidate cross-react in multiple species, including NHPs, minipigs, or rabbits, lower species should be considered first rather than defaulting to NHP as the most relevant species. Rodents (when pharmacologically relevant) are not feasible for repeated

intraocular (IC, IVT, *etc*.) toxicology studies due to ocular anatomical limitations (Rowe-Rendleman *et al.* 2014).

Dose Selection, Dosing Frequency, and Study Duration

From a toxicological standpoint, local intraocular administration offers the promise of the best combination of high local exposure and low systemic exposure. The eye of common laboratory species (*i.e*., NHP, minipig, dog, or rabbit) is large enough to administer the clinically relevant dose volume via a clinically relevant route of administration and allow maximum feasible dose (MFD) administration. Administering the MFD allows the establishment of robust local safety margins to the human dose. Oftentimes, the MFD is achieved by administering two injections of a clinical dose volume. In the case of IVT, MFD is administered as 2x50ul injections administered approximately 10 min apart to allow the intraocular pressure (IOP) to return to baseline values (Dere *et al.* 2021; Bantseev *et al.* 2018). Stand-alone ocular pilot toxicology studies are done on a case-by-case basis and for cause only. When the target and modality are well characterized, information on ocular tolerability can be obtained via limited endpoints such as clinical ophthalmic exams and, in some cases, ocular imaging (i.e., optical coherence tomography (OCT) and fundus photos) as part of pharmacokinetics/pharmacodynamics (PKPD) studies. If a pilot toxicology ocular study is warranted, at least two doses should be administered to mimic periodic dosing in the clinic, as there is limited utility in characterizing ocular tolerability following a single intraocular administration. Dose-ranging (more than a single dose level) may be considered in the pilot toxicology to inform the dose selection and dosing frequency in the Good Laboratory Practices (GLP) toxicology ocular studies. If clinical formulation is available at the time of the pilot toxicology study, it should be used. In the absence of clinical formulation, a research-grade formulation may be considered for use in a pilot toxicology study. As already mentioned, the MFD should be considered a high dose in the pilot toxicology and GLP toxicology studies to establish robust local ocular safety margins to the human dose. The GLP toxicology study should be done with the clinical formulation. Short-duration bridging toxicology studies may be considered if the clinical formulation undergoes significant changes during the clinical development.

Dosing frequency in the pilot toxicology and GLP toxicology ocular studies is guided by the CDP. Differences in the ocular clearance in animals vs. humans are a guiding principle of PK modeling for dosing frequency selection. Dosing both eyes of each animal to the MFD typically allows for the achievement of at least 10-fold systemic margins (Cmax and AUC), as required by ICH S6(R1) for biologics *via* the clinical route of administration (*i.e*., IVT) in an effort to identify

NEW TECHNIQUES AND TECHNOLOGIES TO STUDY VARIOUS ASPECTS OF OCULAR HYPERTENSION/GLAUCOMA

CHAPTER 43

Gene Therapy Approaches and Protocols to Deliver Genes for IOP-Reduction in Animals

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Abstract: The glaucomas represent a heterogeneous group of optic neuropathies that result in progressive optic degeneration characterized by functional and structural impairment of ocular tissues. The two primary damaged ocular tissues in glaucoma are the trabecular meshwork in the front of the eye and the retinal ganglion cells in the back of the eye whose axons form the optic nerve. Elevated intraocular pressure (IOP) is the key risk factor for glaucoma, and drugs lowering IOP are the first line of defense in glaucoma therapy. Although self-administered topical ocular hypotensive eyedrops have been used for glaucoma management, patients encounter several problems, including non-compliance and incorrect drop instillation, especially patients suffering from motor function or coordination dysfunction. Unfortunately, as the majority of patients are asymptomatic, they become indifferent to the urgency of treatment. Even more problematic is that many patients require using multiple types of eye drops, and the likelihood of adherence decreases significantly, leading to worsening of the disease. Therefore, there is an urgent need for long-lasting IOP lowering therapy for glaucoma, and gene therapy offers such a possibility. Here, we describe several gene therapy approaches that are in development and protocols to deliver genes for IOP reduction in glaucomatous eyes.

Keywords: Adeno-associated virus, Gene, Intracameral, Intravitreal, Sonoporation, SiRNA, Therapy.

INTRODUCTION

Glaucoma is the second leading cause of irreversible blindness worldwide, affecting approximately 80 million people, and unfortunately, this number is expected to exceed 111 million by 2040 (Tham *et al.* 2014). It is estimated that there are more than 3 million people in America with glaucoma, with half of them undiagnosed, as glaucoma is frequently relatively asymptomatic until peripheral

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vision deteriorates (Tham *et al.* 2014). It is estimated that the number of US glaucoma patients will reach 7.3 million by 2050 (Vajaranant *et al.* 2012). Sadly, the percentage of undiagnosed cases among Hispanics has reached > 80%, and it has been suggested that 50% of glaucoma patients in the US will be Hispanic by 2050 (Niraj and Joos 2016). Primary open-angle glaucoma (POAG) is the most prevalent form of glaucoma, affecting 90% of the USA's glaucoma patients. POAG is three to four times more common in African Americans than in non-Hispanic whites (Tielsh *et al.* 1991, Marshall 1989, Sommer *et al.* 1991, and Leske *et al.* 1994). Glaucoma is 6 times more common among African Americans than Whites and is 15 times more likely to cause irreversible blindness among them (Friedman *et al.* 2004 and Allison *et al.* 2019). Intriguingly, many of the population are not proactive about vision health, and even when diagnosed, medical intervention is not sought. Medication adherence is of great importance, especially in the early stages of glaucoma. A study has shown that an average of 27% of recently diagnosed POAG patients did not get any type of medical intervention (Stein *et al.* 2008). Further, the first line of treatment *via* self-administered topical ocular hypotensive eyedrops is associated with several issues, including incorrect drop instillation, adherence, and contamination. Davis *et al.* have shown that up to 80% of patients contaminate their eye drop bottle by accidently touching their eye or face, and up to 37% miss the eye completely. In another study, only 10% of glaucoma patients were found to be fully adherent to medications (Friedman 2007). Glaucoma causes an economic burden on patients with an estimated cost of 3 billion annually for the US economy (Rein *et al.* 2006). It appears that the cost of treatment depends on the severity of glaucoma, with early stages costing ~$623 compared to $2511 for severe cases (Lee *et al.* 2006). While it is common for drug therapy to be used as a 'first-option' treatment for glaucoma, surgical intervention is practiced when drug therapy fails. The cost of glaucoma surgery varies with the type of surgery and the area of the country where it is performed.

Potential Therapeutic Genes to Lower Intraocular Pressure

Microarray studies and genetic analysis have examined changes in gene expression in glaucomatous tissue versus nonglaucomatous tissues to identify genes that are either downregulated or upregulated, as well as mutated genes. Genome-wide association studies (GWAS) have identified important loci associated with primary open-angle glaucoma (POAG) risk (Gharahkhani *et al.* 2021). More importantly, the number of single nucleotide polymorphisms (SNPs) associated with glaucoma has continuously uncovered unknown genes that appear to affect aqueous humor (AH) (Abu-Amero *et al.* 2015). In addition, based on the physiology of AH outflow, scientists have examined genes that can alter AH flow rates. Lowering IOP by ~ 25% has been clinically effective in preserving the

vision of glaucoma patients (Heijl *et al.* 2002), and such reduction in IOP may be considered as a standard for developing effective gene therapy for glaucoma.

Gene-knockout to Reduce IOP

The glaucoma association of an SNP identified caveolin-1 (CAV-1), not caveolin-2 (CAV-2), as a potential IOP-lowering therapeutic (Aga *et al.* 2014). Caveolin-1 knockout using short hairpin shRNAs resulted in increased outflow facility for CAV-1 but decreased facility for CAV-2 (Aga *et al.* 2014). On the contrary, Cav-1 knockout mice have shown reduced outflow facility and elevated IOP (Elliott *et al*., 2016; Lei *et al*., 2016; De Ieso *et al*., 2020) due to permeant structural change and, therefore, may not be a good target to lower IOP. Another protein that appears to regulate the cytoskeletal actin network in TM is the GTPase-dependent rho kinase. Rho-kinase inhibitor is the latest IOP-lowering drug approved for glaucoma treatment, which functions to increase AH outflow (Batra *et al.* 2021). The inhibition of Rho-A gene expression using an adenoviral vector carrying a mutant dominant-negative RhoA protein that lacks the GTP binding site (AdhRhoA2) increased the outflow facility (Rao *et al.* 2005 and Vittitow *et al.* 2002). Borras *et al.* 2015 have shown that intracameral injection of scAAV2 expressing the dominant-negative RhoA also lowered IOP in rats at night (Borrás *et al.* 2015). The actin cytoskeleton in the TM can also be directly manipulated through the expression of caldesmon. Caldesmon, an actin-binding protein, can inhibit myosin binding to actin and calcium-dependent smooth muscle contraction. Gabelt *et al.* have shown that the overexpression of caldesmon using adenoviral viruses increased outflow in organ-cultured human and monkey anterior segments (Gabelt *et al.* 2006).

Gene Knockout Using CRISPR-Cas9 to Lower IOP

Clustered regularly interspaced short palindromic repeats (CRISPR) is one of the most exciting techniques that presents promising potential for glaucoma gene therapy targeting the anterior segment of the eye (Sander and Joung 2014). CRISPR-Cas9-mediated genome editing is driven by the binding of protein Cas9 to the targeted site and cleaving the target sequences of DNA. The protein 9 (Cas9) system can achieve efficient and precise gene editing ability, leading to permanent changes of only desired genes and, thus, effective IOP lowering treatment (Wu *et al.* 2020). While CRISPR-Cas9 gene-editing system has been successfully delivered to the retina by electroporation (Wang *et al.* 2014), a novel AAV serotype known as ShH10-AAV carrying the CRISPR cargo can transduce the ciliary epithelium following intravitreal injection. Disruption of aquaporin-1 (AQP1), a water channel involved in aqueous humor production in the ciliary epithelium, lowered IOP in two mouse models of glaucoma, the steroid and the

CHAPTER 44

Protocol for the Delivery of Stem Cells as Trabecular Meshwork

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Abstract: In this chapter, the protocol for the delivery of stem cells as trabecular meshwork cell replacement therapy is described. There are several types of stem cells that have been investigated in ocular regenerative medicine, such as induced pluripotent stem cells, mesenchymal stem cells, tissue-specific stem cells (progenitor cells), and adipose tissue-derived stem cells. These stem cells have the capacity to differentiate into functional cells and have been used in various *ex vivo* and animal models of glaucoma to restore intraocular pressure (IOP) homeostasis and trabecular meshwork (TM) cellularity. The protocol provides a series of steps that can serve as a guide for researchers in the production and use of induced pluripotent stem cells (iPSCs) as cell replacement therapy for the TM, which is damaged and/or lost due to ocular hypertension-induced glaucomatous condition.

Keywords: Intraocular pressure, Induced pluripotent stem cells (iPSCs), Progenitor cells, Stem cells, Trabecular meshwork.

INTRODUCTION

The trabecular meshwork (TM) constitutes the main pathway for the drainage of aqueous humor (AQH) from the eye (Fan *et al*., 2021). Anatomically, the TM is located within the iridocorneal angle (the recess between the cornea and the anterior surface of the attached margin of the iris). The role of the TM is to regulate the outflow of aqueous humor from the anterior chamber of the eye into the adjacent Schlemm's canal. A defect in this function leads to resistance to outflow, elevated intraocular pressure (IOP) (ocular hypertension [OHT]), and the

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risk of glaucoma. Glaucoma is an optic neuropathy and one of the leading causes of vision loss worldwide. It is characterized by irreversible optic nerve damage and progressive vision loss (Fan *et al*., 2021). Studies show that in glaucoma, there is decreased TM cellularity, TM cell loss, abnormal accumulation of extracellular matrix (ECM) components, and increased stiffness of TM (Weinreb and Khaw, 2004; Epstein, 2012). Treatment strategies to reduce IOP include drug therapies that target TM as therapeutics, such as Rho-kinase inhibitors, adenosine agonists, prostaglandin receptor agonists, and potentially inhibitors of SPARC (a fibrotic protein), as well as surgical interventions (Schehlein and Robin, 2019; Chan *et al*., 2019). However, since OHT-induced glaucomatous damage primarily begins in the front of the eye, TM cells endeavor to correct the imbalance in ECM production/deposition by releasing proteases that digest the ECM and then phagocytosing the byproducts, thereby clearing the AQH outflow pathway and lowering IOP. Thus, the use of stem cells to regenerate lost or damaged TM tissue has been proposed as a novel means to treat OHT/glaucoma (Zhou *et al*., 2020; Fan *et al*., 2021; Coulon *et al*., 2022). The purpose of this chapter is to provide a series of steps that can serve as a guide for researchers in the generation and use of induced pluripotent stem cells (iPSCs) to successfully deliver to the TM for TM regeneration and as cell replacement therapy.

EXPERIMENTAL PROTOCOLS

The following procedures were developed according to general considerations for the ethical use of stem cells in research. We focus mostly on methods adapted for human tissue-derived cells for brevity and relevance. Essentially, technology has advanced to a point where almost any somatic cell can be reprogramed into iPSCs by transfecting them with the specific Yamanaka factors (SOX2, KLF4, OCT4, and cMyc) (please see Kumar *et al.* (2020) and references therein for more information). Since the neural crest derivation of TM cells occurs during embryonic development, it was hypothesized that iPSCs could first be induced to differentiate into neural crest cells (NCCs) and then differentiate them into TM cells. Thus, human iPSCs were initially created by reprogramming human dermal fibroblasts, and their properties were determined as previously described (Lin *et al*., 2012). In this study, human iPSCs were differentiated into TM cells via the NCC stage utilizing the conditioned media and ECM derived from cultured primary human TM cells.

Production and use of iPSCs for TM Repair

- Using the differentiated human iPSC-TM cells from the aforementioned procedures, culture them using mTeSR medium supplemented with fibroblast growth factor 2 (FGF2) (final concentration of FGF2 at 100ng/ml) on Matrigel

(Corning)-coated plates minus the feeder cells in an incubator at 37^0C in an atmosphere of 5% CO_2 (Kumar *et al*., 2020).

- The culture medium should be changed as soon as the cells reach 80-90% confluency.
- Use Accutase to dissociate the iPSC colonies, dilute the enzyme in the plate with fresh medium (5-10 ml) lacking any growth factors, centrifuge the mixture, and discard the medium containing the enzyme.
- Resuspend cells into a fresh mTeSR medium and dispense into A549 cell-derived ECM-coated plates for 3 days (Kumar *et al*., 2020).
- Switch the medium after 3 days to N2B27 medium (DMEM/F12 (1:1) supplemented with 2% B27, 1% N2, 100X Glutamax, 100U/ml penicillin, and 100mg/ml streptomycin with 100 mM Rho kinase inhibitor Y27632 (to induce NCC differentiation) for 10 days.
- Upon establishing a pool of NCCs, differentiate them further into TM cells (see below).
- Following the above procedures, dissociate the NCCs with Accutase as mentioned above and seed onto new TM ECM-coated culture plates. The ECM should be created by lysing >90% confluent TM cells with 0.02 N ammonium hydroxide and 0.05% Triton X-100 for five minutes, decanting the fluid, and storing the plates at -20°C until needed.
- Use TM cell-conditioned culture medium (DMEM/F12 (1:1) with 10% FBS) to induce the cells over 10–14 days. Stain samples of cells to check for the appearance of some TM-cell membrane/cytosol markers such as tissue inhibitor of matrix metalloproteinase-3 (TIMP3), aquaporin-1 (AQP-1) and chitinase 3 like 1 (CHI3L1), thus indicating differentiation into TM cells. Additional checks on the cell identity may include responsiveness to a corticosteroid such as dexamethasone, which would induce ECM and myocilin production and induce cross-linking of actin networks detectable by immunofluorescence studies.
- The iPSC-TM cells can then be transplanted into the anterior eye chamber of animal model systems by intracameral injection to observe whether they integrate into the existing TM structure.

Induction of TM Cells from Mesenchymal Stem Cells

The protocol used for the induction of TM cells from mouse mesenchymal stem cells is described by Nadri *et al.* (2013).

- Isolate bone marrow mononuclear cells (BMMC) by aspiration from the femur and tibia of 2-month-old mice using density gradient centrifugation.
- Suspend the cells in Dulbecco's modified Eagle's medium (DMEM) containing 2 μM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20% heat-inactivated fetal bovine serum.

CHAPTER 45

Isolation and Characterization of Trabecular Meshwork Stem Cell Exosomes from the Conditioned Medium

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Abstract: Exosomes are nanovesicles (30-200nm) whose cargo includes proteins, RNA of different types, DNA, lipids, and small molecules. The exosome-based therapy is gaining significance in modern medicine due to the signal transduction capabilities and non-immunogenic properties of exosomes. In regenerative medicine, stem cell-derived exosomes are employed as an alternative in cell therapies. Currently, there are more than 35 ongoing clinical trials worldwide employing exosomes in various disease conditions, including two eye diseases. However, the efficacy of these nanovesicles in glaucoma therapy still remains unexplored. Trabecular meshwork (TM) is a tiny, porous tissue located in the iridocorneal angle of the eye that maintains intraocular pressure (IOP) homeostasis. The adult stem cells are located in the non-filtering region and are drastically reduced in glaucomatous conditions (Sundaresan *et al.*, 2021). Our previous TM stem cells (TMSC) transplantation studies on a human organ culture of anterior segment (HOCAS) model (Iswarya *et al.*, ISSCR 2021) and several other studies on animal models (Yun *et al.*, 2018; Xiong *et al.*, 2021,) promise the application of TMSCs in glaucoma therapy. To evaluate the efficacy of the TMSC exosomes as an alternative to cell-free therapy for glaucoma, we optimized a few *in vitro* assays, which give insights into the properties of isolated exosomes. This chapter describes protocols to isolate exosomes by ultracentrifugation, characterization by nan-

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oparticle tracking analysis (NTA), Western blot analysis, and transmission electron microscopy (TEM). Further, the functional efficacy of the exosomes was evaluated by *in vitro* wound healing assay and an anti-oxidant assay.

Keywords: Anti-oxidant, Conditioned medium, Exosomes, Glaucoma, *In vitro* wound healing, Nanoparticle tracking analysis (NTA), Trabecular meshwork stem cells, Transmission electron microscopy (TEM), Ultracentrifugation, Western blotting.

INTRODUCTION

Exosomes are nanovesicles (30-200nm) that contain a variety of biomolecules, such as proteins, lipids, and nucleic acids. The exosomes gained importance in therapeutic applications because of their size and ability to cross blood barriers as well as other barriers such as the blood-brain barrier (BBB), blood-cerebrospinal fluid barrier (BCSFB), blood–lymph barrier (BlyB), blood–air barrier (BAB), stromal barrier (SB), blood–labyrinth barrier (BLaB), blood-retinal barrier (BRB), and placental barrier (PB). Non-immunogenicity and the ability to transduce signals make them ideal nanocarriers. They can be engineered to carry specific cargo and deliver to specific targets. The exosomes mediate cell-to-cell communication and many other cellular processes through their cargo. The exosomes are excellent biomarkers as well. Mesenchymal stem cell (MSC) exosomes have gained significant attention in recent years as they can replace the need for MSC-based therapy. Currently, there are more than 35 ongoing clinical trials using MSC exosomes worldwide, including two eye diseases, which emphasize the efficacy of exosomes in cell-free therapy (https://www.clinicaltrials.gov/). However, there are no reports on the exosome-based therapy for glaucoma.

Trabecular meshwork (TM), a tiny, porous tissue located at the iridocorneal angle of the eye, is responsible for intraocular pressure (IOP) homeostasis. The stem cells for TM (TMSCs) are located in the non-filtering region (Sundaresan *et al.*, 2019), which is drastically reduced in glaucomatous conditions (Sundaresan *et al.*, 2021). Transplantation of TMSCs in cell loss human organ culture of anterior segment (HOCAS) model (Iswarya *et al.*, ISSCR 2021- unpublished data) and several other studies on animal models (Yun *et al.*, 2018; Xiong *et al.*,2021) enhanced TM cell proliferation and reducted in IOP, indicating the possibility of developing a stem cell therapy for glaucoma. Therefore, there is a huge potential for the use of TMSC exosomes for the treatment of glaucoma.

The purpose of this chapter is to describe our optimized, simple–to-use, and reliable methods to isolate and characterize exosomes from TMSC-derived

conditioned medium since the use of proper isolation method is essential to maintain the integrity and functional competence of the exosomes.

TMSC Culture Establishment

Materials

Trabecular meshwork from donor eyes, 35mm dish, collagenase A (Roche, Germany), serum-free medium, pipettes, 15 ml falcon tubes, Stem Cell Growth Medium (SCGM- Opti-MEM, 5%FBS (Gibco- Thermofisher Scientific, USA), 200µg/ml calcium chloride, 0.08% chondroitin sulfate, 20µg/ml ascorbic acid, 100µg/ml bovine pituitary extract (Sigma-Aldrich, Darmstadt, Germany), 10ng/ml EGF (Invitrogen- Thermofisher Scientific, USA), pipettes, and tips.

Procedure

Sample: For TM tissues, whole globes (not suitable for transplantation) and corneoscleral rims (after removal of the cornea for transplantation) were obtained from the eye banks of Aravind Eye Hospitals.

- TM was carefully removed from the rims using fine forceps and digested with 4mg/ml collagenase A for 2 hours at 37°C in a CO_2 incubator (Stamer *et al.*, 1995).
- After incubation, the reaction was stopped by adding a complete medium and centrifuged at 1500g for 5 minutes.
- The cells were suspended in SCGM and cultured for 3-4 weeks (Du *et al.*, 2012) with the media change in alternative days.
- When the cells reached confluency, they were trypsinized and passaged in T75 flasks to obtain adequate cells for conditioned medium collection (Fig. **1**).

Conditioned Medium Collection

Materials

SCGM, Opti-MEM (31985-070- Thermofisher Scientific, USA), T75 flasks, serum-free medium, 10ml serological pipettes, culture grade PBS, exosome depleted FBS (System Biosciences, California, USA), and 50 ml falcon tubes.

Procedure

- Seed 2.1 x10^6 TMSCs in T75 flasks and maintain them for two days to reach confluency.
- Upon confluency, remove the medium with a sterile serological pipette and wash the adhered cells with PBS five times gently.

CHAPTER 46

Protocol for Single-Cell RNA-Sequence Analysis of Genetic Models of Glaucoma

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Abstract: We used two genetically modified mouse models to elucidate the role of different cell types in the initiation and progression of glaucoma. Retinal ganglion cell death has been strongly associated with glaucoma development, and several key genes expressed in certain cell types have been identified. This includes the astrocyte-specific *ABCA1* gene and the *P2RY6* gene, which are found in non-pigmented epithelial cells of the ciliary body. This section describes the protocols for performing scRNA-sequencing to elucidate crucial mechanisms in cell populations and data analysis of sequencing data using the 10X Genomics platform and Seurat package in R.

Keywords: ABCA1, Astrocytes, Eye, Glaucoma, Glia, Intraocular pressure, Mouse models, Normal tension glaucoma, P2Y6 receptors, Protocol, Retina, Retinal ganglion cell, Single-cell RNA-sequencing.

INTRODUCTION

The purpose of this chapter is to describe the process of dissecting the murine retina and isolating cells for use in single cell-RNA sequencing to examine the progression of glaucoma from specific cell populations (Shinozaki *et al*., 2022). These procedures allow us to identify the molecular pathways and biological events in cell-line-specific mouse models of glaucoma and their effects on surrounding cell populations that contribute to the glaucoma-like phenotype observed in these mice. The use of these mice models can be combined with existing treatments for glaucoma to elucidate novel therapeutic strategies or prognostic markers to diagnose glaucoma early.

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Glaucoma is an age-related disease that affects over 70 million people worldwide, with approximately 10% presenting with full blindness (Quigley & Broman, 2006). Blindness in the most common forms of glaucoma arises due to increased intraocular pressure (IOP) in the anterior chamber of the reduced drainage of the aqueous humor through the trabecular meshwork, resulting in progressive damage to retinal neurons. The focal point of mechanical IOP-associated stress is the optic nerve head, which is enriched with retinal ganglion cell (RGC) axons (Burgoyne *et al*., 2005). The subsequent loss of this population leads to irreversible injury to axons in the optic nerve head and at the lamina cribrosa (Fig. **1**). This is followed by the loss of RGC soma. Rarer forms of the disease, such as normal tension glaucoma (NTG), are also caused by the progressive loss of RGCs due to ischemia, glutamate neurotoxicity, inflammation, and hypoxia, but without high IOP in patients (Shen *et al*., 2023).

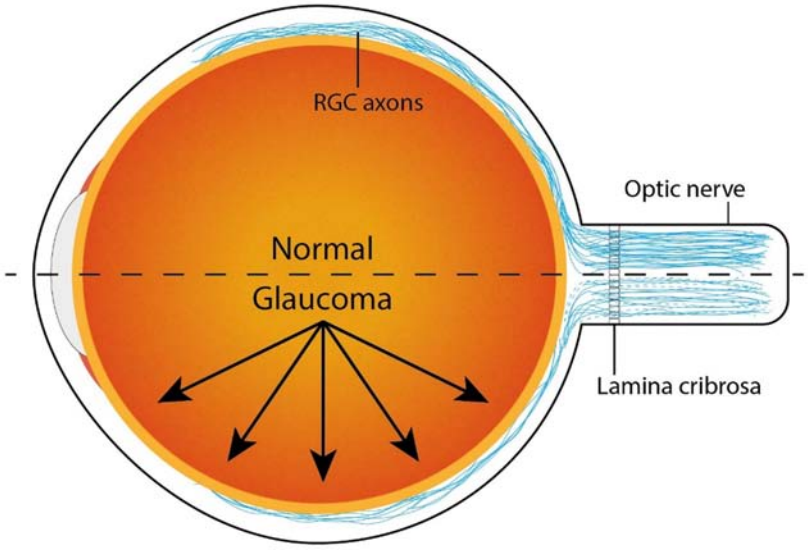

Fig. (1). Schematic representation of the eye depicting loss of RGCs. In the normal eye, RGC function and status is maintained. With the development of glaucoma and increased intraocular pressure (arrows), RGC function is destroyed and cells undergo apoptosis, leading to thinning of the nerve fibre layer.

Most *in vivo* glaucoma models induce IOP or other symptoms of glaucoma in an attempt to replicate the disease, while few genetic knockout models exist. This is, in part, due to the complexity of the disease progression. However, genetic mouse models are still crucial in elucidating specific mechanisms that may initiate or play a role in glaucoma development.

Several candidate driver genes of glaucoma have been described in genome-wide association studies (GWAS). At least four large-scale GWAS from independent,

international cohorts have identified single nucleotide polymorphisms clustered by the ATP-binding cassette transporter 1 (*ABCA1*) gene on chromosome nine (Chen *et al*., 2014; Gharahkhani *et al*., 2014, 2021; Hysi *et al*., 2014). The *ABCA1* gene encodes a membrane-associated cholesterol and phospholipid efflux pump and has already been implicated in other age-related retinal diseases such as AMD and Alzheimer's disease (Lewandowski *et al*., 2022; Lupton *et al*., 2014). Indeed, patients with Alzheimer's disease present with a two- to three-fold increased risk of developing glaucoma. Furthermore, other studies have suggested a link between cholesterol levels and glaucoma (Kang *et al*., 2019; Posch-Pertl *et al*., 2022).

An *Abca1*$^{-/-}$ mouse model was constructed from a DBA1/lacJ mouse strain using homologous recombination to excise 910 base pairs from exon 17-22 using methods from the study by Hamon (2000). A reduction of the retinal ganglion marker, *Brn3a,* and elevation of apoptotic cells in the absence of IOP elevation in 12-month-old mice suggested that *Abca1*$^{-/-}$ mice are a suitable model for normal tension glaucoma.

These promising observations warranted further refining of the model. ABCA1 was found to co-localize in cells expressing GFAP, indicating specific expression in astrocytes. Additional studies have since confirmed *Abca1* expression in astrocytes (Y. Zhang *et al*., 2014; Y. Zhang & Wallace, 2015). Thus, astrocyte-specific, conditional *Abca1*$^{-/-}$ mice were generated by crossing *ABCA1* floxed mice with GFAP-Cre mice (Timmins *et al*., 2005). Similar phenotypes to non-specific *ABCA1*$^{-/-}$ mice were observed, along with reduced visual response to multifocal electroretinograms. These findings confirmed the importance of *ABCA1* expression in retinal astrocytes and how their dysregulation can contribute to the apoptosis and degeneration of RGCs.

While *Abca1*$^{-/-}$ mice appear to be useful models for normal-tension glaucoma, a model for the most common variant of the disease, primary angle-closure glaucoma, is yet to be identified. A candidate for primary angle-closure glaucoma became apparent with the discovery of elevated nucleotide levels in the aqueous humor of human patients with glaucoma (Li *et al*., 2011; Markovskaya *et al*., 2008; X. Zhang *et al*., 2007). These molecules bind to P2 receptors, which are divided into P2X and P2Y subfamilies, and they are found to be expressed in tissues within the eye such as the cornea, ciliary processes, trabecular meshwork, photoreceptors, and ganglion cells (Pintor *et al*., 2004; Shinozaki *et al*., 2023).

Extensive studies on the P2X and P2Y receptor families highlighted P2Y6 as a strong nominee for glaucoma-like phenotypes *in vivo* (Shinozaki *et al*., 2017). Administration of UDP in wild-type C57BL/6J mice reduced IOP by suppressing

CHAPTER 47

Protocols for Studying Nanotubes and their Role in Human Ocular Cell Communication in the Anterior Chamber of the Eye

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Abstract: Communication between cells can be accomplished through the release and receipt of soluble chemical messenger molecules *via* endocrine and/or paracrine mechanisms. Additional connections can be made *via* the transmission of signals across gap junctions amongst neighboring cells. In recent years, however, a novel means of cellular communication, tunneling nanotubes (TNTs), has been identified that involves physical contact between cells such as trabecular meshwork (TM) cells in the anterior chamber of the eye aqueous humor drainage system that regulates intraocular pressure (IOP). The current chapter describes such TNTs in human TM cells to study them and to gain a better understanding of their role in the physiology and pathophysiology of human IOP regulation. Comparisons of TNTs in glaucomatous and normal TM cells can shed light on how TM cell communication or dysfunctions in TNTs may negatively impact IOP regulation and cause ocular hypertension.

Keywords: Actin cytoskeleton, Cellular communication, Filopodia, Glaucoma, Myosin-X, Trabecular meshwork, Tunneling nanotube.

INTRODUCTION

A heterogeneous population of trabecular meshwork (TM) cells possessing smooth muscle cells, endothelial cells, and structural features are present in the aqueous humor (AQH) drainage system in the anterior chamber of the eye (Abu-Hassan *et al*., 2014; Acott *et al*., 2020). The TM is inexorably connected to the Schlemm's canal (SC), which is an extension of the veinous micro-vessels through which the AQH finally drains as it exits the anterior chamber. This TM/SC pathway is a pressure-dependent system that regulates the intraocular pressure (IOP) by controlling the AQH drainage. When this outflow route of AQH egress is blocked due to the accumulation of unwanted proteins, lipids, and

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cellular debris, the IOP rises and damages the retinal-optic nerve-brain structures and reduces axonal connectivity, thereby starting the process of glaucomatous damage and inducing visual impairment. If this elevated IOP (ocular hypertension [OHT]) is not reduced by drugs or surgery and is allowed to keep rising, more and more damage is exerted to the optic nerve and retinal ganglion cells. After decades of slow but progressive vision loss, the patient can become blind unless the OHT is diagnosed and timely treated.

Under normal circumstances, cells within the TM and SC monitor their environment and the IOP and somehow regulate the efflux of AQH to maintain a steady IOP. How these cells achieve IOP homeostasis has been a mystery until the recent discovery of nanometer-sized tunneling nanotubes (TNTs) through which cells transfer signaling chemicals and organelles (mitochondria, hormones, transmitters, micro-RNAs, neurotrophins, enzymes, endosomes, lysosomes, *etc.*). Such a communication system has evolved in the eye, presumably since the traditional means of diffusional delivery of extracellularly secreted signaling molecules in the anterior chamber is not optimally possible. Here, the unidirectional AQH fluid flow from the ciliary body, where it is produced through the anterior chamber and out the TM/SC outflow system in the eye, would otherwise be constantly diluting the compounds and the resident time on the various cells and their receptors/ion-channels/transporters would be severely limited. Hence, communication through the TNTs should largely be unaffected by the fluid flow. Indeed, TNTs connect different TM/SC cells to permit intercellular communication and permit transfer of agents directly between the cells. These nano-scaled membranous tubes are 50-200 nm in diameter (Gerdes *et al*., 2013), and they play a prominent role in intercellular trafficking between TM cells and to and from SC cells (Marzo *et al*., 2012; Keller *et al*., 2017, 2019). TNTs are a unique form of filopodia that advance from cell to cell, which, upon fusion, create a hollow connectome through which proteins and other molecules can travel between the cells by an “actin-myosin driven” system. In the second format of the cellular information transfer mechanism, cells can combine, and then a nanotube forms when the cells migrate away in different directions (Sun *et al*., 2019a). Myosin-10 (Myo-X) is important and intimately involved in filopodia and TNT formation and cargo transfer. The myosin head binds to actin filaments, and the tail, which contains a special protein, helps transport cargo to the filopodia tips (Sun *et al*., 2019a,b). In this manner, sensing of elevated IOP by TM cells probably triggers intra- and inter-cellular communication *via* such nanotubes to promote protease release to help digest excess ECM within the TM/SC and thus keep the outflow pathway open for AQH to egress and hence regulate IOP. These coordinated activities help lower IOP and maintain it at a desired and optimal level. Using the protocols described herein, the research scientists can observe the existing and newly formed TNTs and compare and contrast the dimensions, the

number, and potentially the functions of the TNT in normal and glaucomatous TM and SC cells *via* live-cell imaging techniques (Fig. **1**). Protocols to permit the isolation, study, and quantification of various TM cell functions, including TNTs, are provided below.

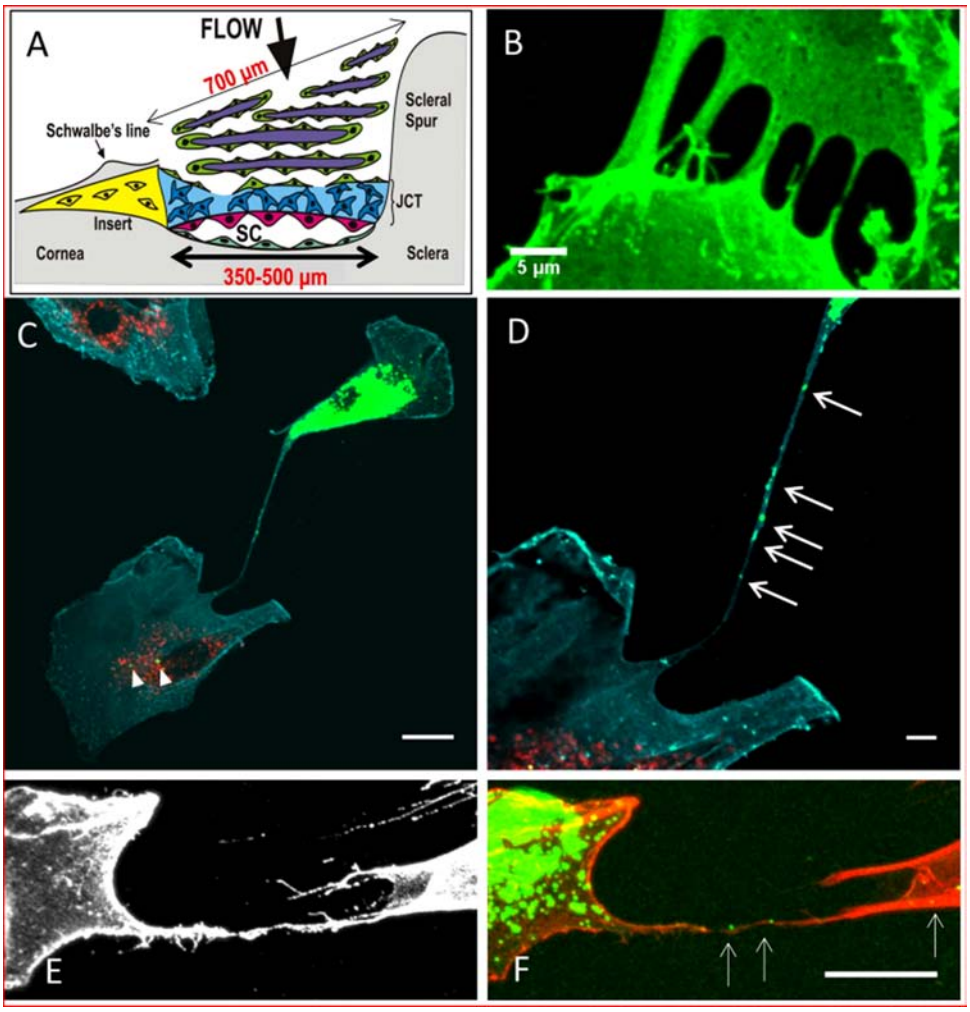

Fig. (1). (A) Schematic of a cross-section of the TM, with the regions and approximate dimensions of the tissue indicated. TM cells (green); corneoscleral beams (purple), the JCT region (blue); the inner wall cells of Schlemm's canal (pink); the putative stem cell insert region (yellow); and Schlemm's canal. (B) TM cells immunostained with CD-44 to label the cell membrane. Filopodia from neighboring cells appear to touch and fuse to form a tube. Scale bar: 5 um. (C) In coculture experiments, Dio-labeled vesicles (green) were present in a DiD-labeled (red) cell (arrowbeads). Cyan: CD44; scale bar: 20 µm. (D) At higher magnification, DiO-labeled vesicles are clearly visible (arrows) within a long cell process connecting the two cells. Scale bar: 5 µm. (E) CD44 immunostaining (gray) of a TNT connecting two TM cells. (F) DiO-labeled vesicles (green; arrows) are clearly visible within the SiR-actin-labeled cell process (red). Scale bar. 20 µm. Keller *et al*., (2017).

CHAPTER 48

Protocol for Aqueous Humor Metabolite Measurements for Glaucoma Research

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Abstract: Ultra-high-performance liquid chromatography-mass spectrometry is commonly used in the untargeted analysis of metabolites within mammalian aqueous humor samples. Aqueous humor is a transparent fluid that bathes the anterior segment of the eye to nourish the cornea and lens. Aqueous humor also assists in the maintenance of intraocular pressure. In glaucoma, aqueous humor flow is impeded, leading to ocular hypertension and optic nerve damage. The application of metabolomics to study aqueous humor allows for advancements in knowledge of the molecular mechanisms in glaucoma. In this chapter, we put forth a protocol using hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass spectrometry to accurately identify metabolomic data of the aqueous humor samples. The purpose of this chapter is to provide an extraction and identification method of mammalian aqueous humor metabolites using liquid chromatography-tandem mass spectrometry.

Keywords: Aqueous humor, Glaucoma, HILIC, LC-MS/MS, QC Samples, Untargeted metabolomics, Ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS).

INTRODUCTION

Glaucoma is a group of progressive diseases that leads to optic nerve degeneration. It is the leading cause of irreversible blindness worldwide, with about 70 million people affected by primary open-angle glaucoma—the most common type (Zhang *et al*., 2021). With life expectancy increasing globally, res-

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earchers have estimated that by 2040, glaucoma will affect 111.8 million patients (Tham *et al*., 2014). Up to half of glaucoma cases remain undetected due to their symptomless nature, which has resulted in biomarker advancements to aid in early diagnosis and potential therapy treatments (Barbosa Breda *et al*., 2020).

Metabolomics is defined as the analytical measurement of all known and unknown metabolites within a fluid, tissue, or cell (Han *et al*., 2020). The recent emerging approach of metabolomics has allowed researchers to identify biomarkers within diseases, study the natural effects of drugs, and explore the metabolic pathways performed by a specific disease (Pietrowska *et al*., 2017). Mass spectrometry-based analysis is highly effective when identifying metabolites within biological samples. The analyses are typically performed by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS/MS). When using LC-MS/MS, metabolites are subject to contamination and background noise. Therefore, it is essential that sample preparation and extraction are done efficiently, as well as to ensure the performance of the instrument through quality controls, internal standards, and extraction blanks (Jauregui *et al*., 2023). Pooled quality control samples (Pooled QCs) are a pool of all samples within the study used to monitor the instruments' stability and the quality of the data being produced. Blank samples like blank mobile phases and extraction blanks provide information on the origin of the metabolites being identified. Both of these data filtering techniques reduce the size of the dataset by limiting the inclusion of non-biological metabolites (Ivanisevic and Want, 2019). For optimal results in identification, two ion modes are applied when looking for results in metabolite identification. In positive mode, the ions analyte is charged *via* protonation, whereas in negative ion mode, the analyte is charged *via* deprotonation (Banerjee and Mazumdar, 2012). In complexes such as plasma, urine, or tissue, hundreds to thousands of metabolites can be identified within the database. It is important to acknowledge that polar compounds do not bind well on the reverse phase column compared to the HILIC column, which is specifically created for separating highly polar compounds (Jauregui *et al*., 2023). The data results are most efficient when using both HILIC and reversed phase methods to identify both water-soluble charged metabolites and semi-polar metabolites (Najdekr *et al*., 2019).

In this chapter, we present an untargeted mass spectrometry method utilizing HILIC to identify metabolites of interest in aqueous humor samples from mammalian species. We present here the example of UHPLC- MS/MS instruments. However, these protocols are useful across the board with any LC-MS/MS system with additional adjustments to the specific instruments used.

EXPERIMENTAL PROTOCOLS

The following procedures were performed in compliance with the guidelines of the American Association for Laboratory Animal Science for the use of animals and adhered to the Statement for the Use of Animals in Ophthalmic and Visual Research from the Association for Research in Vision and Ophthalmology (ARVO).

MATERIALS AND CHEMICALS

- Ammonium formate
- 200 Mm Ammonium acetate
- Acetonitrile (ACN)
- Formic Acid
- Methanol (MeOH)
- Extraction solvent (for extraction from plasma): 1 mL stock solution of MeOH: ACN (v/v, 1:1)

ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

Instruments

- Thermo Scientific™ Vanquish™ Horizon UHPLC.
- Thermo Scientific™ Q ExactiveTM Orbitrap™ Mass Spectrometer coupled to a heated electrospray ionization source (HESI).
- Thermo Scientific™ Xcalibur™
- Vacuum centrifuge
- Sonicator

ULTRA HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMNS AND VIALS

- AccucoreTM 150 Amide HILIC Column (150 mm x 2.1 mm, 2.6 µm, Thermo Scientific™).
- HPLC vials (catalog number: 6PRV11-03FIVP) and caps (catalog number: 11PSN(B)-8RT1).

CHAPTER 49

Protocols for Performing CRISPR Gene Editing in Anterior Chamber Eye Cells

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Abstract: The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology has revolutionized genetics and opened up new possibilities for research and therapy. The CRISPR system is composed of single guide RNA (sgRNA) and the endonuclease (Cas9), which can be combined into a ribonucleoprotein complex (RNP). RNP can be used to target and cut specific DNA regions. The introduction of foreign DNA at the cut's site allows for precise genome editing, such as insertion, deletion, or substitution of specific genes. This powerful technology has been widely adopted in many fields, including basic research, drug discovery, and biotechnology. Its ability to efficiently edit genes has made it an essential tool for studying gene function, developing new therapies for genetic diseases, and improving crops and livestock. This chapter will discuss the utility of CRISPR/Cas9 and sgRNA using trabecular meshwork Cells (GTM3 cells), highlighting the limitations and potential alternative methods. We will also describe the T7 endonuclease I (T7EI) assay, a protocol used to confirm gene editing.

Keywords: Clustered regularly interspaced short palindromic repeats, Delivery methods, Guide RNA, Gene modification.

INTRODUCTION

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system is a revolutionary gene editing technology that has revolutionized the field of genetics and created many exciting possibilities for research and therapeutic development. (Doudna & Charpentier, 2014). The CRISPR/Cas system is a bacterial immune system composed of short repetitive sequences and DNA fragments from previously encountered foreign DNA that provides adaptive im-

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munity in most prokaryotic organisms (Barrangou *et al*., 2007; Jinek *et al*., 2012). Recently, this system has been adapted for use as a gene-editing tool and has transformed the field of genetic research and opened up the possibility of treating a wide range of genetic diseases (Doudna & Charpentier, 2014).

The CRISPR/Cas9 system is a highly versatile and efficient gene-editing tool that uses a guide RNA (gRNA) to direct the Cas9 endonuclease to specific target sequences in the genome. Upon binding to the target site, Cas9 cleaves the DNA, allowing for the insertion, deletion, or substitution of specific genetic sequences with high precision (Hsu *et al*., 2014; Doudna & Charpentier, 2014). This system has been widely adopted in various fields, including basic research, drug discovery, and biotechnology, and has facilitated significant advancements in these areas (Sander & Joung, 2014).

However, the delivery of CRISPR/Cas9 to target cells remains a challenge due to the large size of the system and the potential toxicity associated with some delivery methods (Wang *et al*., 2016). Several approaches have been developed to overcome this limitation, including viral, non-viral, and physical methods of delivery, which can be used to deliver plasmids, ribonucleoprotein complex (RNPs), or other components of the CRISPR system (Wang *et al*., 2016; Chen *et al*., 2019).

Here, we will review the various methods for delivery of CRISPR/Cas9 and gRNA to ocular cell lines (We will use the GTM3 cell line as an example) and ways to confirm gene editing, highlighting the limitations of each approach and suggesting possible alternative methods.

EXPERIMENTAL PROTOCOLS

The following procedures were approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee review board.

Cell Selection: GTM3

Transformed GTM3 cell line (Pang, I. H *et al*., 1994) were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 1% L-glutamine (Gibco, Life Technologies), and 1% penicillin-streptomycin (Gibco, Life Technologies).

Cell Seeding: Forward Versus Reverse Seeding

Forward seeding and reverse seeding are two methods used to introduce cells into a culture. In forward seeding, cells are added to the culture at a low density and allowed to grow and divide over time to reach confluence. This method is

commonly used when growing cells for long-term experiments or cell-based assays (Freshney, 2010). In reverse seeding, cells are added to the culture at a high density, often close to confluence, and then allowed to attach and spread. This method is commonly used for short-term experiments or for cells that do not adhere well to the culture surface (Masters *et al*., 2018). The choice between forward and reverse seeding depends on the specific research goals and the studied cells' characteristics. For example, forward seeding may be preferred for long-term studies or for cells that require time to adjust to the culture environment, while reverse seeding may be used for short-term studies or for cells that do not adhere well (Estrada *et al*., 2017).

Selection of CRISPR System Components

Selection of gRNA

Single-guide RNA (sgRNA) is a crucial element of the CRISPR-Cas9 gene-editing system, and its design is essential for the efficiency and specificity of the system. The following guidelines are used for designing efficient and specific sgRNA for gene editing, and several online tools are available to help with this process:

a. **Choose the target site:** The target site should be conserved across different alleles, specific to the gene of interest, and located near a protospacer adjacent motif (PAM) sequence that is required for Cas9 binding and cleavage.
b. **Avoid off-target effects:** To avoid off-target effects, sgRNA should not target sites with homology to non-targeted genes. Various online tools such as CRISPOR (Haeussler *et al*., 2016) and E-CRISP (Heigwer *et al*., 2014) are available to predict off-target effects and assist with sgRNA design.
c. **Optimize sgRNA design:** The design of the sgRNA should be optimized for efficiency and specificity, with consideration of factors such as the length of the sgRNA, the GC content, and the location of the PAM sequence. Various online tools such as Benchling (https://benchling.com/crispr) and CHOPCHOP (https://chopchop.cbu.uib.no) are available to help with sgRNA design and optimization.

By following these guidelines and utilizing online tools, one can design efficient and specific sgRNA for gene editing, allowing for precise and targeted modifications to the genome.

Selection of Appropriate Cas9 Enzyme

The CRISPR-Cas system for gene editing requires the selection of the appropriate Cas9 enzyme, which is a critical step. Among various Cas enzymes, Cas9 from

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Drs. Sharif and Ohia have brought together a wonderful collection of protocols to study disease mechanisms and drug target discovery/validation for glaucoma. This is a valuable reference guide for researchers in the ocular hypertension/glaucoma field

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