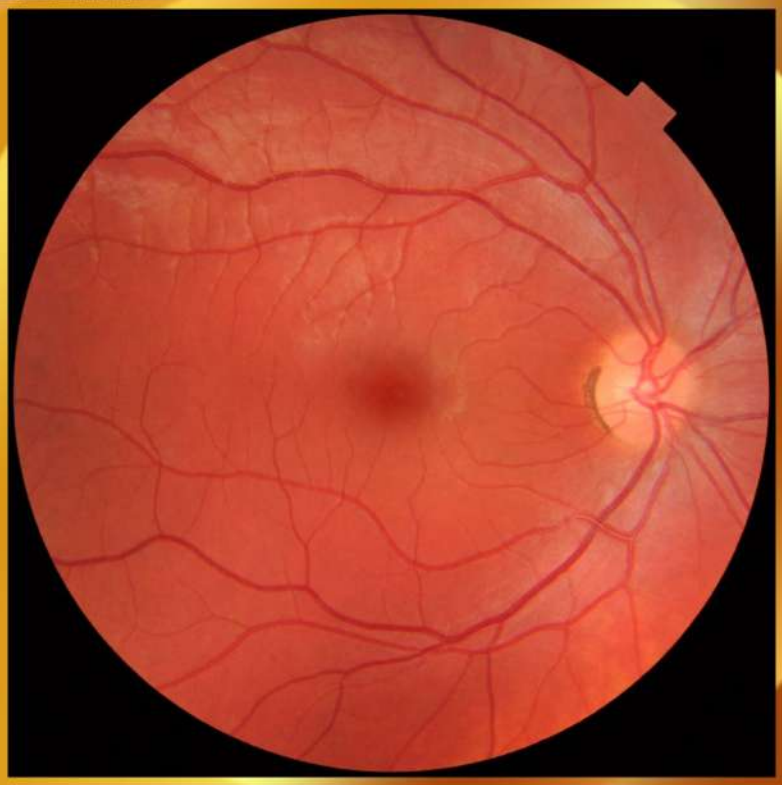
ISSN 2475-1278

Openventio
PUBLISHERS

THE OPHTHALMOLOGY

Open Journal

December 2020 | Volume 4 | Issue 1 |



www.openventio.org

ISSN 2475-1278

THE OPHTHALMOLOGY
Open Journal

Openventio
PUBLISHERS

CONTENTS

ISSN 2475-1278

THE OPHTHALMOLOGY Open Journal

Openventio PUBLISHERS

Observational Study

Micropulse Transscleral Cyclophotocoagulation: Our Experience

Syed S. Ahmad, MS[1,*]; Shuaibah A. Ghani, MS[2]; Ghuncha Khatoon, BUMS[3]; Sumera Sagheer, BUMS[3]; Juwairiya Ilyas, BUMS[3]

[1]*Ibn Sina Academy of Medieval Medicine and Sciences, Aligarh, Uttar Pradesh, India*
[2]*University Malaysia Sabah, Kota Kinabalu, Malyaysia*
[3]*Ajmal Khan Tibbiya College, Aligarh, Uttar Pradesh, India*

[*]***Corresponding authors***
Syed S. Ahmad, MS
Ibn Sina Academy of Medieval Medicine and Sciences, Aligarh, Uttar Pradesh, India; E-mail: syedshoebahmad@yahoo.com

Article information
Received: February 10th, 2020; **Revised:** March 20th, 2020; **Accepted:** March 28th, 2020; **Published:** April 24th, 2020

Cite this article
Ahmad SS, Ghani SA, Khatoon G, Sagheer S, Ilyas J. Micropulse transscleral cyclophotocoagulation: Our experience. *Ophthalmol Open J*. 2020; 4(1): 1-4.
doi: 10.17140/OOJ-4-120

ABSTRACT

Introduction
Traditionally, ciliary body destruction has been used to treat uncontrolled intraocular pressure (IOP) following maximally tolerable medical therapy. This is due to the large number of complications seen with this procedure. However, recently a new technique of sub-threshold laser or micropulse laser, is able to provide selective destruction of the ciliary body in a controlled manner. This avoids most of the complications seen with other modalities. We have performed a small case descriptive pilot study to assess the effectiveness of micropulse transscleral cyclophotocoagulation (MP-TSCPC) in lowering IOP.

Methods
This pilot study was conducted on four patients in the age range 55-70-years with intractable glaucoma. Two patients had primary angle closure glaucoma, one-each had steroid-induced glaucoma and neovascular glaucoma. Mean baseline IOP was 32±2.4 mmHg. Mean number of glaucoma medications were 2.5±1.5. All patients underwent 180° MP-TSCPC. Absolute success was defined as IOP<20 mmHg without acetazolamide.

Results
Following the procedure the patients were followed-up at days 1,7,30 and 90. At the last follow-up of the study, mean IOP was 18.2±1.2 mmHg in all four patients. Mild anterior chamber inflammation was the only complication noted. Mean number of glaucoma medications reduced to 1.5±1.0 following the procedure. Thus, absolute success was achieved in all patients.

Conclusion
This small pilot study validates other studies which show effectiveness of MP-TSCPC as an efficient and safe procedure to lower IOP. This procedure can be used over a wide variety of cases, though the indications for such procedures are still evolving. More extensive and long-term studies will clarify the position of this procedure in our glaucoma management practices.

Keywords
Glaucoma; Micropulse laser; Cyclophotocoagulation.

INTRODUCTION

Glaucoma is a multifactorial neurodegenerative disorder. There are a number of risk factors involved in the pathogenesis of glaucoma. These include raised intraocular pressure (IOP), increasing age, race, family history of glaucoma, certain medical conditions such as diabetes mellitus and others. However, the only risk factor which can be controlled at present is IOP. This can be lowered either by reducing aqueous production by the ciliary body or increasing outflow through the trabecular meshwork (conventional pathway) or uveo-scleral tract (unconventional pathway). Certain drugs are able to act through these two aforementioned mechanisms. Glaucoma filtering surgeries and glaucoma drainage devices aim to decrease IOP by increasing aqueous outflow. Aqueous production can be decreased by utilizing surgical and laser methods for the destruction of ciliary body. These cyclodestructive procedures have been in clinical use for many years and provided mixed results.

Ophthalmol Open J. 2020; 4(1): 1-4. doi: 10.17140/OOJ-4-120

Cyclodestruction was previously reserved for refractory glaucoma.[1] This technique was utilized after maximally tolerable medical therapy was ineffective and the patient had pain, poor visual acuity or poor visual potential or other complications due to uncontrolled IOP. It has also been advocated when conjunctival scarring prevents any further surgery on the eye or when patients are unfit or refuse surgery. The rationale for limiting cyclodestruction to such above mentioned cases was the unpredictable nature of the procedure. In eyes with satisfactory visual potential, more conventional methods such as trabeculectomy or lens extraction were preferred.[2]

Conventional cyclodestructive laser procedures use continuous laser delivery to destroy the ciliary body. Therefore, the site of aqueous production, namely the ciliary epithelium is damaged. IOP falls as a direct consequence of reduced aqueous production. However, these lasers also cause collateral damage to tissues in and around the ciliary body. This has the potential of excessive reduction of aqueous humor production leading to ocular hypotony or even sympathetic ophthalmia. Such unpredictable results of these cyclodestructive procedures have precluded their use in eyes with some degree of visual potential.

Recently, a new technique of micropulse transscleral cyclophotocoagulation (MP-TSCPC) has been introduced. The potential advantage of this procedure is focused, repetitive ("on-off") delivery of laser energy to the pigmented ciliary epithelium. This avoids collateral damage and more controlled IOP reduction is possible. This technique has expanded the indications for cyclophotocoagulation in eyes which had previously been excluded from these procedures.

We undertook this small pilot descriptive observational study to assess the effectiveness of MP-TSCPC. Being a relatively new technique very few studies regarding this modality have been published. We have provided here the results of our study as well as a review of the currently available literature.

MATERIALS AND METHODS

The inclusion criteria for our study were patients with poor visual potential despite maximally tolerable medical therapy. Pain was not a requisite for including the individual in the study. Infact, only one patient in the study had ocular pain from the high IOP. Exclusion criteria were patients who had good visual potential or who were unfit for the procedure or those who refused the procedure.

Four patients in the age group 55-70-years were enrolled for the procedure. There were two males and two female patients. Two patients had primary angle closure glaucoma, one had steroid-induced glaucoma and one had neovascular glaucoma. The mean baseline IOP was 32±2.4 mmHg. Written informed consent was obtained from all patients. Absolute success was defined as IOP below 20 mmHg without oral acetazolamide. The study was performed after clearance from the Institutional Ethical Board and with consideration to the tenets of Declaration of Helsinki (Table 1).

All procedures were performed on the same day by one of the authors (SSA). Peri-bulbar anesthesia was used for the procedure using 2% lignocaine. Under aseptic precautions adequate exposure of the upper limbus was made. We performed MP-TSCPC using the Iridex MP3 machine, provided to us on loan. The laser was set to 2000 mW; total duration of the procedure was 1.6 millisecond (ms), including 0.5 ms "on" time and 1.1 ms "off" time, with 31.33% duty cycle. Only the upper 180° of the limbus was treated, avoiding the 3- and 9- o'clock area where the ciliary neurovascular structures are present. The probe was kept about 3 mm posterior from the limbus and perpendicular to the globe. In a "painting fashion" the probe was passed from one end to the other. Following the procedure an antibiotic-steroid ointment was applied and the eye padded for 24-hours.

The patients were seen in the clinic the next day and followed-up on day 7, day 30 and at day 90 after the procedure. During each visit parameters such as visual acuity and IOP were recorded. The symptoms and signs were noted by attending clinicians. At three months after the procedure the mean IOP was 18.2±1.2 mmHg; thus, absolute success was achieved in all four patients. No serious complications were noted during follow-ups. The only complication noted was mild anterior chamber inflammation which resolved within 30-days after the procedure. The number of glaucoma medications reduced from a pre-procedure mean of 2.5±1.5 to 1.5±1.0 post-operatively.

DISCUSSION

Historically, cyclodestruction by non-penetrating diathermy was first used by Weve.[3] Subsequently, Vogt[4] modified the technique so that a penetrating diathermy probe could be introduced through the sclera and destroy the ciliary processes. Experimental use of radium to destroy the vascular supply of ciliary body was reported by Haik et al.[5] The technique led to lens damage and was not used

Table 1. *Patient Characteristics*

S.No	Age (Years)	Sex	Type of Glaucoma	Mean preop IOP (mmHg)	Laterality	Mean postop IOP (mmHg)	Visual Acuity
1	64	Female	PACG	32	Right eye	16	HM
2	55	Male	SIG	34	Left eye	18	CF 1m
3	70	Male	NVG	36	Left eye	20	NPL
4	62	Female	PACG	30	Right eye	18	HM

PACG=Primary angle closure glaucoma; SIG=Steroid induced glaucoma; NVG=Neovascular glaucoma; HM=Hand movement; CF=Counting fingers; NPL=No perception to light.

clinically. Berens et al[6] used cycloelectrolysis by using low-frequency galvanic current. High-intensity focused ultra-sound was initially conceptualized by Purnell et al.[7] The most recent application of high-intensity focused ultrasound (HIFU) has been ultrasound cycloplasty. This procedure allows selective coagulation of the ciliary body and avoids possible damage to the adjacent ocular structures. In addition, stimulation of supra-choroidal and trans-scleral portions of the uveoscleral outflow pathway has recently been proposed as possible adjunctive mechanisms in reducing IOP.[8]

Finger et al[9,10] used trans-scleral microwave radiation to produce heat-induced ciliary body destruction. Weekers et al[11] used xenon arc photocoagulation to achieve transscleral cyclodestruction (TS-CPC). Vucicevic et al[12] used a ruby laser to achieve the first laser-induced transscleral cyclophotocoagulation. Later neodymium yttrium aluminum garnet (Nd:YAG) laser and finally diode laser were successfully incorporated as procedures for TSCPC.

Traditional TSCPC lasers, such as the diode laser, deliver continuous energy to the ciliary body. This has the potential to damage collateral tissues, leading to complications such as hypotony, visual acuity changes, sympathetic ophthalmia and phthisis bulbi. MP-TSCPC delivers repetitive, short bursts of laser energy in an "on-off" manner. The pulses of light are emitted in the infrared region (810 nm) and strongly absorbed by melanin present in the pigmented ciliary epithelium. During the "on" phase the thermal photocoagulative effect of the laser destroys the ciliary body. During the "off" phase the adjacent non-ciliary structures are allowed to cool, protecting them from thermal damage. This reduces the complications seen in traditional TSCPC. The mechanism of TSCPC is apparently through damage to ciliary body, increased uveoscleral outflow, inflammatory effect on the ciliary body, and activation of cellular biochemical cascades which cause decreased IOP.[13-16]

There are only a few studies on the efficacy of MP-TSCPC available in current literature. In a study of MP-TSCPC in refractory glaucoma conducted by Tan et al[17] a 30% reduction in IOP in 72.7% patients and reduction in glaucoma medications from 2.1 to 1.3 at 18-months of follow-up was reported. Aquino et al[18] has compared MP-TSCPC with continuous wave-TSCPC (CW-TSCPC) in 48 patients with refractory glaucoma. At 18-months of follow-up 52% in the MP-TSCPC group and 30% in the CW-TSCPC group were able to maintain an IOP of 6-21 mmHg (30% reduction from preoperative level). Mean number of glaucoma medications reduced from 2 to 1.[19] Kuchar et al[20] has reported his study of MP-TSCPC on 19 patients with advanced glaucoma. Atleast 20% lowering of IOP was achieved in 73.7% patients after 60-days of follow-up. Mean glaucoma medications reduced from 2.6 to 1.9. In another study by Emanuel et al[21] consisting of 84 eyes, MP-TSCPC achieved success in all patients by reduction of IOP from a mean of 27.7 to 11.1 mmHg (59.9% reduction). Glaucoma medications reduced from mean 3.3 to 2.3 after 12-months of follow-up. Gavris et al[22] in his study of MP-TSCPC in refractory glaucoma reported mean IOP reduction at one week in 60.3% patients and in 33.4% patients at one month. There was mean reduction in glaucoma medications by 0.71 at one month of follow-up. Williams et al[23] performed their study in which 79 patients with refractory glaucoma underwent MP-TSCPC. Following the procedure, IOP between 6-21 mmHg was achieved in 75% cases at 3-months and in 66% at 6-months. Mean number of glaucoma medications reduced from 2.3 to 1.5 at last follow-up. Our study provided better results compared to the above mentioned reports probably due to the small size of cohort and shorter follow-up. We plan to undertake a larger and longer evaluation after this pilot study.

CONCLUSION

Micropulse transscleral cyclophotocoagulation appears to be a useful addition to our armament of glaucoma management. The indications for the procedure are evolving. Presently, the treatment is being offered to patients in order to reduce the number of glaucoma medications or reduce ocular discomfort from raised IOP. We performed this small case descriptive pilot study to determine the protocol and efficacy of this procedure. As this was a pilot study we restricted our intervention to only those eyes who had limited visual potential. In this study IOP reduction was achieved in all patients with minimal complications at three months of follow-up. However, looking at the limited number of patients and types of glaucomas encountered during the study we recommend larger studies with longer follow-up and with different types of glaucoma. Such studies will provide us better understanding of the procedure and the possibility to extend it to other patients who have traditionally been kept out of the purview of cyclodestructive procedures. The exact status of MP-TSCPC at this time remains equivocal.

LIMITATIONS

Since the number of patients in our study are very small and restricted to a few types of glaucomas it is not possible to extrapolate the results to all types of glaucomas. Secondly, our follow-up was short (3-months) and so long-term results of the procedure are not available yet.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Allingham RR, Damji KF, Freeman S. *Shields' Textbook of glaucoma*. 6th ed. Philadelphia, USA: Wolters Kluwer/Lippincott Williams and Wilkins; 2012.

2. Martin KR, Broadway DC. Cyclodiode laser therapy for painful, blind glaucomatous eyes. *Br J Ophthalmol*. 2001; 85: 474-476. doi: 10.1136/bjo.85.4.474

3. Weve H. Die zyklodiatermie das corpus ciliarebei glaukom [In: German]. *Zentralbl Ophthalmol*. 1933; 29: 562-569.

4. Vogt A. Cyclodiathermy puncture in cases of glaucoma. *Br J Ophthalmol*. 1940; 24: 288-297.

5. Haik GM, Breffeilh LA, Barber A. Beta irradiation as a possible therapeutic agent in glaucoma. *Am J Ophthalmol*. 1948; 31: 945-952. doi: 10.1016/0002-9394(48)92523-9

6. Berens C, Sheppard LB, Duel AB. Cyclo electrolysis for glaucoma. *Trans Am Ophthalmol Soc*.1949; 47: 364-382.

7. Purnell EW, Sokollu A, Torchia R, Taner N. Focal chorioretinitis produced by ultrasound. *Invest Ophthalmol Vis Sci*. 1964; 3: 657-664.

8. Lim KS. Ultrasound cycloplasty in glaucoma – mechanisms of action and their possible impact on intraocular pressure. *European Ophthalmic Review*. 2017; 35: 9. doi: 10.17925/EOR.2017.11.01.35

9. Finger PT, Smith PD, Paglione RW, Perry HD. Transscleral microwave cyclodestruction. *Invest Ophthalmol Vis Sci*. 1990; 31: 2151-2155.

10. Finger PT, Moshfeghi DM, Smith PD, Perry HD. Microwave cyclodestruction for glaucoma in a rabbit model. *Arch Ophthalmol*. 1991; 109: 1001-1004. doi: 10.1001/archopht.1991.01080070113047

11. Weekers R, Lavergne G, Watillon M, Gilson M, Legros AM. Effects of photocoagulation of ciliary body upon ocular tension. *Am J Ophthalmol*. 1961; 52: 156-163. doi: 10.1016/0002-9394(61)91110-2

12. Vucicevic ZM, Tsou KC, Nazarian IH, Scheie HG, Burns WP. A cytochemical approach to the laser coagulation of the ciliary body. *Bibl Ophthalmol*. 1969; 8: 467-478.

13. Ma A, Yu SWY, Wong JKW. Micropulse laser for the treatment of glaucoma: A literature review. *Surv Ophthalmol*. 2019; 64: 486-497. doi: 10.1016/j.survophthal.2019.01.001

14. Kosoko O, Gaasterland DE, Pollack IP, Enger CL. Long-term outcome of initial ciliary ablation with contact diode laser transscleral cyclophotocoagulation for severe glaucoma. *Ophthalmology*. 1996; 103: 1294-1302. doi: 10.1016/s0161-6420(96)30508-3

15. Pastor SA, Singh K, Lee DA, et al. Cyclophotocoagulation: a report by the American Academy of Ophthalmology. *Ophthalmology*. 2001; 108: 2130-2138. doi: 10.1016/s0161-6420(01)00889-2

16. Schlote T, Derse M, Rassmann K, Nicaeus T, Dietz K, Thiel HJ. Efficacy and safety of contact transscleral diode laser cyclophotocoagulation for advanced glaucoma. *J Glaucoma*. 2001; 10: 294-301. doi: 10.1097/00061198-200108000-00009

17. Tan AM, Chockalingam M, Aquino MC, Lim ZI, See JL, Chew PT. Micropulse transscleral diode laser cyclophotocoagulation in the treatment of refractory glaucoma. *Clin Exp Ophthalmol*. 2010; 38: 266-272. doi: 10.1111/j.1442-9071.2010.02238.x

18. Aquino MC, Tan AM, Loon SC, See J, Chew PT. A randomized comparative study of the safety and efficacy of conventional versus micropulse diode laser transscleral cyclophotocoagulation in refractory glaucoma. *Invest Ophthalmol Vis Sci*. 2011; 52: 2609.

19. Aquino MC, Barton K, Tan AM, Sng C, Li X, Loon SC, Chew PT. Micropulse versus continuous wave transscleral diode cyclophotocoagulation in refractory glaucoma: A randomized exploratory study. *Clin Exp Ophthalmol*. 2015; 43: 40-46. doi: 10.1111/ceo.12360

20. Kuchar S, Moster MR, Reamer CB, Waisbourd M. Treatment outcomes of micropulse transscleral cyclophotocoagulation in advanced glaucoma. *Lasers Med Sci*. 2016; 31: 393-396. doi: 10.1007/s10103-015-1856-9

21. Emanuel ME, Grover DS, Fellman RL, et al. Micropulse cyclophotocoagulation: Initial results in refractory glaucoma. *J Glaucoma*. 2017; 26: 726-729. doi: 10.1097/IJG.0000000000000715

22. Gavris MM, Olteanu I, Kantor E, Mateescu R, Belicioiu R. IRIDEX MicroPulse P3: Innovative cyclophotocoagulation. *Rom J Ophthalmol*. 2017; 61: 107-111.

23. Williams AL, Moster MR, Rahmatnejad K, et al. Clinical efficacy and safety profile of micropulse transscleral cyclophotocoagulation in refractory glaucoma. *J Glaucoma*. 2018; 27: 445-449. doi: 10.1097/IJG.0000000000000934

ISSN 2475-1278

THE OPHTHALMOLOGY
Open Journal

Openventio
PUBLISHERS

Brief Research Report

Anti-Platelet-Derived Growth Factor Receptor-Beta Therapy Does Not Trigger Retinal Endothelial Cell Toxicity

Zachary K. Goldsmith, PhD[1,2#]; Andrew S. Irvine, BS[1#]; Matthew W. Wilson, MD, FACS[1,3*]; Vanessa M. Morales-Tirado, MS, PhD[1,2,4*]

[#]*Both Authors Contributed Equally to the Work*

[1]*Department of Ophthalmology, Hamilton Eye Institute, University of Tennessee Health Science Center, Memphis, TN, USA*
[2]*AbbVie Bioresearch Center, Worcester, MA, USA*
[3]*Department of Surgery, St. Jude Children's Research Hospital, Memphis, TN, USA*
[4]*Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA*

[*]***Corresponding authors***
Matthew W. Wilson, MD, FACS
Department of Ophthalmology, Hamilton Eye Institute, The University of Tennessee Health Science Center, 930 Madison Ave, Room 471, Memphis 38163, TN, USA; Tel. (901) 448-5883; E-mail: mwilson5@uthsc.edu

Vanessa M. Morales-Tirado, MS, PhD
Department of Ophthalmology, Hamilton Eye Institute, The University of Tennessee Health Science Center, 930 Madison Ave, Room 471, Memphis 38163, TN, USA; E-mail: vmoralestirado@gmail.com

Article information
Received: October 28th, 2020; **Revised:** November 9th, 2020; **Accepted:** December 1st, 2020; **Published:** December 2nd, 2020

Cite this article
Goldsmith ZK, Irvine AS, Wilson MW, Morales-Tirado VM. Anti-platelet-derived growth factor receptor-beta therapy does not trigger retinal endothelial cell toxicity. *Ophthalmol Open J*. 2020; 4(1): 5-10. doi: 10.17140/OOJ-4-121

ABSTRACT

Background
Retinoblastoma (Rb) is a highly angiogenic tumor, for which anti-vascular endothelial growth factor (VEGF) therapies have shown limited success in clinical setting. Recent investigations demonstrated upregulation of ancillary axis including the platelet-derived growth factor (PDGF) when VEGF is inhibited. This illustrates the need for novel therapeutics. Previous work from our lab showed inhibition of the platelet-derived growth factor receptor-beta (PDGFR-β) by imatinibmesylate (IM), inhibited Rb cells proliferation *in vitro*. Novel therapies ideally are tumor-specific, leaving normal non-cancerous cells a stroma to perform their homeostatic functions. Rb treatments induce apoptosis of the retinal endothelial cells, causing the release of pro-inflammatory cytokines and chemokines to the microenvironment.

Aims
We investigated the role of the PDGFR-β in the tumor microenvironment and how inhibition of this signaling pathway, as a potential targeted therapy, impacts angiogenesis in human retinal microvascular endothelial cells (hRECs), specialized neurons arborizing the retinal microvasculature.

Results
Our results demonstrated that inhibition of the PDGFR-β signaling pathway by IM affects the proliferation of the Rb cells, but not hRECs. PDGFR-β signaling is not required for hRECs angiogenic activity, although it reduces the percentage of VEGF-A-producing cells.

Conclusion
These results illustrate a lack of functional activity PDGFR-β signaling in hRECs and points to a more tumor-specific therapeutic option. This is of critical importance as success of treatment also depends on the ability of the normal tissues to remain healthy after sensitization and/or killing of the Rb tumor.

Keywords
Retinoblastoma; Retinal endothelial cells; Ocular oncology; Imatinib mesylate.

INTRODUCTION

Approximately 300 children are diagnosed each year in the United States with retinoblastoma (Rb), the most common intraocular cancer among childhood malignancies.[1,2] The severity of the disease depends primarily on the size and location of the primary tumor. If dissemination occurs Rb can spread throughout the retinal to the vitreous region, central regions of the brain, to the bones and bone marrow. Metastases significantly reduce the survival rate. Treatments can include enucleation, systemic or local chemotherapy, focal therapy, radiation therapy and in rare instances stem cell transplantation.[3] These treatments emphasize on the child's life first, while vision and preservation of the eye are of secondary concern.[4]

Disruption of the blood-retinal barrier (BRB), leaky vessels and high-levels of angiogenic responses including vascular endothelial growth factor (VEGF) and the active platelet-derived growth factor receptor-beta (PDGFR-β) signaling, are characteristics of Rb.[5-7] Therefore, targeting the tumor and the angiogenic response may provide an alternative therapeutic strategy to this disease. In the last decade, Rb research has shifted to focus more on identifying and successfully targeting new pathways that are: 1) tumoricidal, 2) safer for healthy cells necessary for homeostasis and, 3) capable of being administered locally. In our pursuit to develop novel Rb therapeutic strategies we recently identified the PDGFR-β as being highly active in Rb disease.[5] We targeted the PDGF-PDGFR-β signaling pathway using imatinib mesylate (IM, akaGleevec® from Novartis), a protein-tyrosine kinase inhibitor approved to treat acute lymphoblastic leukemia (ALL) in adults and children that are Philadelphia chromosome (summarized in)[8] positive, among other malignancies, and showed *in vitro* reduction of Rb growth, invasion, and survival in an AKT-, MDM2-, and NF-κB-dependent manner.

Current therapies against Rb act on both the tumor cells and the healthy, non-malignant, cells within the stroma. We previously demonstrated melphalan-induced upregulation of ICAM-1 and apoptosis in retinal microvascular endothelial cells.[9,10] Apoptosis of these cells release pro-inflammatory cytokines and chemokines to the microenvironment. In this study, we investigated *in vitro* the effects of disruption of the PDGF-PDGFR-β signaling pathway on primary human retinal microvascular endothelial cells (hRECs) cellular proliferation and function prior to transitioning into pre-clinical assessment of the PDGF-PDGFR-β signaling pathway in Rb. Our work demonstrated that disruption of this signaling pathway is limited to reducing Rb survival without affecting hRECs viability and organization. This work supports our hypothesis that anti-PDGFR-β therapy could be a tumor-specific therapy for use in children afflicted with Rb.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions

Primary human retinal microvascular endothelial cells (hRECs, ACBRI 181) were purchased from Applied Cell Biology Research Institute (Cell Systems, Kirkland, WA, USA). Cells were cultured using four different conditions: untreated, recombinant human Platelet-Derived Growth Factor (rhPDGF, 10 ng/mL), Imatinib Mesylate (IM, 10 μM), and the combination of rhPDGF+IM. Y79 (ATCC, HTB-18) Rb cells[11] were purchased from American Type Culture Collection (ATCC) (Manasas, VA, USA) and cultured as described above.

Cell Proliferation Assay

Cell proliferation studies were performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, USA) as before.[12] Briefly, 1.0×10^4 UM cells per well were cultured in the conditions described above. CellTiter reagent was added at a concentration of 20 μL per 100 μL volume per well at specific time points of 0-, 48-, 72-, and 96 hrs after culture. Cells were incubated at 37 °C for 2 hrs before absorbance was read at 490 nm using a 96-well plate reader. Values expressed as mean±SEM, n=3 with 4 replicates. Statistical analysis done using Prism Graph Pad.

qPCR Analysis

RNA from 1.0×10^6 hRECs was extracted following the Qiagen-miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) manufacturer's recommendations. Synthesis of cDNA was performed using the SuperScript® VILO™ (complementary deoxyribonucleic acid) cDNA Synthesis Kit (Life Technologies, Grand Island, NY, USA). Following manufacturer's directions, we used 100 ng of ribonucleic acid (RNA) combined with the Reaction Buffer and the Enzyme Mix. We used the following Human TaqMan® Gene Expression Assays: HPRT1 (Hs02800695_m1), PDGFRB, and FLT1, all from Life Technologies (Grand Island, NY, USA). A final volume of 10 μL was loaded into each well after combination of TaqMan® Universal Master Mix, cDNA, primers and Nuclease Free water. Plates were run using Roche® Light Cycler 480 and data were analyzed using the Comparative ΔCT Method as before.[12,13] Values expressed as mean±SEM, n=3 with 4 replicates.

Tube Formation Assay

hRECs were cultured on Reduced Growth-Factor Matrigel (BD Biosciences, Bedford, MA, USA) in complete media (10% FBS, Cell BioSystems) or low serum (0.2% FBS). Additional culture conditions included rhPDGF and IM. Images were taken at both 6- and 18 hrs, respectively using a Nikon C1 confocal microscope using 4x objective. Results are representative of two independent experiments; three fields were taken per group at both 10x (data not shown) and 4x.

Flow Cytometry Analyses

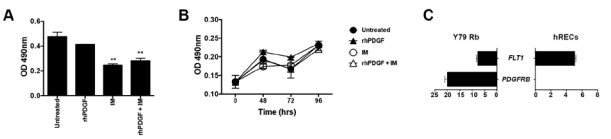
Monocultures of Y79 Rb, hRECs and co-cultures of Y79 Rb and hRECs (Rb:hREC) were treated using the conditions described above. Cell cultures were harvested at 24 hrs, fixed in PBS/2% paraformaldehyde, and labeled with anti-human PDGFR-β APC or with anti-VEGF-A AF700 (BioLegend, San Diego, CA, USA). Data acquisition done using a Bio-Rad ZE5 Cell Analyzer (aka YETI, Propel Labs, Fort Collins, CO, USA); analysis done using FlowJo vX.0.5 (Tree Star). Each analysis done on a minimum of 50,000 events.

RESULTS

Cell Proliferation of Human Retinal Endothelial Cells is Independent of PDGF-PDGFR-β signaling

We first measured Y79 proliferation using the tyrosine kinase inhibitor (TKI) imatinib mesylate (IM), a known inhibitor of PDGFR. We had previously identified 10 μM as the most efficacious concentration.[5] Inhibiting the PDGFR-β signaling with IM significantly reducedthe proliferation of Y79 Rb cells at 72 hrs (Figure 1A). As a physiological control, we stimulated Y79 Rb cells with recombinant human PDGF-BB (rhPDGF), which we discovered to be highly abundant in the tumor microenvironment of Rb.[5] To investigate the role PDGF-BB may have on hRECs and to test if disrupting the PDGF-PDGFR-β signaling pathway by IM may be toxic to hRECs, we cultured hRECs in the same conditions as Y79 Rb cells. We tested proliferation over 96 hrs using MTS cellular proliferation assay and measured no changes in cellular proliferation (Figure 1B). These results illustrate how hREC proliferation occurs independent of PDGF-PDGFR-βsignaling. Next, we investigated the expression levels of PDGFRB to measure receptor activity. We harvested mRNA from hRECs and Y79 cells to measure the expression of FLT1 (VEGFR1) and PDGFRB relative to HPRT1, an endogenous control. The mRNA expression of FLT1 was higher in hRECs compared to Y79 Rb cells. However, PDGFRB mRNA expression was not detected (Figure 1C) in hRECs, while high expression was uncovered in Y79 Rb controls. Taken together, these results demonstrate how targeting of PDGF-PDGFR-β signaling will not have anti-proliferative effects in hRECs, as they do not express PDGFRB.

Figure 1. *hREC Proliferation Occurs Independent of PDGF-PDGFRb Signaling*



*hY79 cells or hRECs were cultured in the presence or absence of either PDGF-BB (10 ng/mL) or the PDGFR-β inhibitor imatinibmesylate (IM, 10 μM). (A) Assessment of Y79 cellular proliferation at 72 hrs the presence of either PDGF-BB or IM using the concentrations described above. (B) Assessment of hREC proliferation over 96 h in the presence of either PDGF-BB or IM using the concentrations described above.(C) qPCR analysis was done on both cell types to measure mRNA levels of both FLT1 and PDGFRB.All experiments done with N=3 and each sample done in triplicates.All results in represent mean ±SEM; **p<0.005.*

Disruption of the PDGF-PDGFR-β signaling Pathway Does not Affect Angiogenic Activity in hRECs

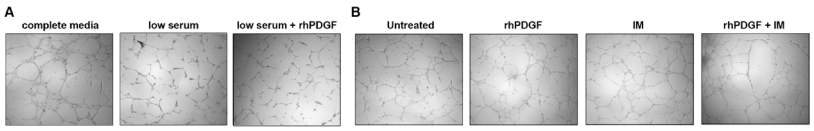
To test the hypothesis that anti-PDGFR-β treatment will not affect the normal, non-pathological, angiogenic activity of hRECs, we cultured hRECs on reduced-growth factor extracellular matrices under the following conditions: complete media (with 10% FBS), low serum (0.2% FBS), and low serum+rhPDGF (10 ng/mL). Non-pathogenic organization of hRECs display complex tubular structures similar to blood vessels (Figure 2A, left).Under stressful conditions, simulated through serum starvation, hRECs do not readily organize into these branched structures, as shown in Figure 2A, middle. Next, we tested if the PDGF-PDGFR-β signaling could restore hRECs ability of tube formation. Results in Figure 2A, right demonstrate this signaling pathway cannot restore hRECs angiogenic activity *in vitro*, defined by the tube formation assay.

As a next step, we tested if the PDGF-PDGFR-β signaling pathway is capable of promoting or stimulating tube formation. hRECs were all cultured in complete media and under the following conditions: untreated, stimulated with rhPDGF (10 ng/mL), IM (10 μM), and rhPDGF+IM. We found no morphological changes in hREC vascular organization (Figure 2B) across the different cell culture conditions. Ultimately, these results indicate hRECs angiogenic function is independent of PDGF-PDGFR-β signaling.

PDGFR-β Contributes to hREC VEGF-A Production

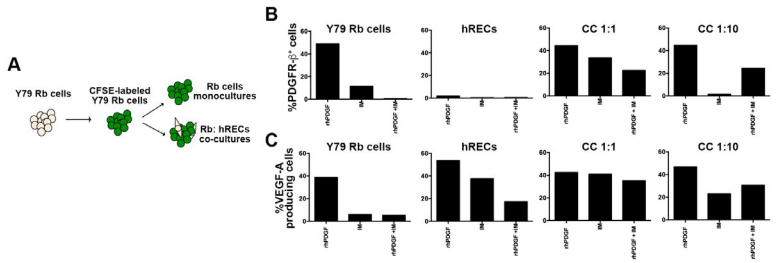
To evaluate cellular interactions and potential role(s) PDGF-PDGFR-β signaling may play in the Rb tumor microenvironment, we set up a co-culture system composed of Y79 Rb cells and hRECs using the same conditions described above. Y79 Rb cells were labeled with the non-toxic dye carboxyfluorescein succinimidyl ester (CFSE) to trace the cells during single cell analyses, while leaving the hRECs unlabeled (Figure 3A). Co-cultures of Rb:hRECs were done using two different ratios, 1:1 and 1:10, to examine potential cell number-dependent roles. Flow cytometry analysis for the evaluation of the percentage of PDGFR-β$^+$ cells showed differences between Y79 Rb cells and hRECs. About half of the Y79 Rb cells display immune positivity for PDGFR-β, while a minimal percentage for hRECs (Figure 3B, top left), confirming our mRNA results in Figure 1. In our co-cultures, we measured reductions in the percentage of cells expressing PDGFR-β after

Figure 2. *PDGF-PDGFR-β signaling is Unable to Initiate Tube Formation*



hRECs were cultured using the following conditions: (A) complete media (10% FBS), low serum (0.2% FBS), low serum in the presence of rhPDGF (10ng/mL); (B) untreated (complete media), rhPDGF, IM(10 μM) or the combination of rhPDGF + IM on reduced growth factor Matrigel. Imageh using a Nikon C1 microscope and 10X (above) and 4X (data not shown) objectives.All experiments done in quadruplicate. Images above are representative of field of view.

Figure 3. *Treatment with IM Reduces hREC VEGF Production*



Y79 and hREC co-cultures were generated. (A) Schematic representation of experimental design. Y79 cells were labeled with CFSE to distinguish them from hRECs. Cells were plated alone or in specific ratios (Rb:hREC) to measure cellular interactions. All cultures were treated with rhPDGF (10 ng/mL) or imatinib mesylate (10 μM). (B) Assessment of PDGFRβ+ by flow cytometry. (C) Identifying the percentage of VEGF-A producing cells (VEGF-A+) using flow cytometry. N=2 due to lack of hREC viability; all flow cytometry analyses done with minimum of 50,000 events.

IM treatment, as shown in Figure 3B, top right.

Next, we examined the percentage of VEGF-A+ cells in Rb cells and hRECs after disruption of the PDGF-PDGFR-β. There was a reduction in the percentage of vascular endothelial growth factor-A (VEGF-A)-producing cells in both mono- and co-cultures after disruption of the PDGF-PDGFR-β signaling pathway (Figure 3C). Collectively, our results show a reduction in Y79 Rb cells expressing PDGFR-β+ and a reduction in VEGF-A-producing cells in both Rb tumor cells and hRECs.

DISCUSSION AND CONCLUSION

Retinoblastoma (Rb) remains the most common intraocular malignancy afflicting children. While survival rates are typically above 95% in the United States, clinical management is challenging in developing countries. New mechanisms for drug delivery have been developed in recent decades, specifically those of super-selective intra-ophthalmic artery chemotherapy (SSIOAC) and intravitreal chemotherapy (IVT).[14,15] These new treatment mechanisms have garnered much attention into increasing ocular salvage. Still, they often have severe side effects for the pediatric population including ischemia, neutropenia, a higher risk for development of a secondary malignancy, and blindness.[14,16-18]

In recent years emerging efforts at the development of novel therapeutics for the control of Rb tumor cells, while being safe for non-malignant cells for homeostasis, have increased. Therapeutic interventions at controlling VEGF have not succeed, suggesting other factors in addition to VEGF play pivotal roles in Rb. We recently identified the PDGFβ-PDGFR-β signaling pathway as an active pathway in Rb. Our work demonstrated a novel-signaling pathway for potential targeted therapy that could improve ocular survival in advanced Rb. While our initial studies focused on targeting of the tumor cells, here, we investigated the effects of pharmacological disruption of this pathway in microvascular retinal endothelial cells, key players in the blood-retinal barrierhighly affected by many ocular diseases including Rb and diabetes retinopathy. All of these as necessary steps prior to transitioning into pre-clinical evaluation.

Previous work from our team[9,10] identified how melphalan and carboplatin, two commonly administered chemotherapies to treat Rb, increase retinal endothelial cell death and inflammation.[9] A recent report discovered superselective intraophthalmic artery chemotherapy (SSIOAC) administration, while allowing for higher concentrations of localized drug delivery, does affect the contralateral eye with concentrations of drug in plasma being similar to those of systemic (or intravenous) administration.[19] As endothelial cells are necessary to supply the healthy neural retina with nutrients *via* blood supply, significant loss of these cells can result in neurodegeneration and, ultimately, vision loss. These endothelial cells are also the primary component of the BRB that prevents cellular infiltration into the healthy retina and use of an anti-PDGFR-β therapy could help maintain this essential structure.[20,21] Our study demonstrates the pro-apoptotic effects of IM are Rb cell specific, as hRECs proliferation is not affected. In contrast to human umbilical vein endothelial cells (HUVEC), which are widely used in *in vitro* studies of endothelial cells, hRECs cannot signal through the PDGFR-β. This was demonstrated in our studies by genomic and flow cytometry analyses. Of note, we measured a reduction in the %VEGF-A+cells in the hRECs and Y79 monocultures. Work from Pennock and colleagues[22] suggested VEGF-A could signal through the PDGFR-β to promote cell viability during hypoxic conditions. Studies in our laboratory showed no difference in VEGFA, VEGFR mRNA in Y79Rb cells (data not shown) when the PDGFβ-PDGFR-β signaling pathway is disrupted. Collectively, this work suggests anti-PDGFR-β therapy could be an Rb tumor-specific therapy.

The present study investigated the role of the PDGF-PDGFR-β signaling pathway in hRECs. As a functional readout of disruption of this signaling pathway we used a genomic (PDGFRB mRNA expression), flow cytometry (percentage of PDGFR-β+ cells and VEGFA-producing cells), and angiogenic (tube formation assay) approaches. We are cognizant of the limitations of the study as we focused on a small cohort of hRECs properties *in vitro*. We utilized the primary hRECs, which are isolated after medical enucleations to link our cellular results to the translation of human disease. These are considered "*truly microvascular cells from the retina*",[23] in contrast to macrovascular cells from human umbilical vein or human artery. Literature reports demonstrated the presence of PDGFR-β in human umbilical vein endothelial cells (HUVEC), which contrast to the results presented in this work using hRECs,

highlighting the need to use the appropriate models to translate the work. Further studies aim at investigating endothelial cell leukostasis and toxicity *in vivo* using a Rb xenograft model developed by our colleagues.[24] Histopathological findings will address ocular effects after *in vivo* disruption of the PDGF-PDGFR-β signaling pathway and will allow for more in-depth analyses at cellular interactions within the microenvironment.

Children with Rb face cosmetic and psychological challenges if undergo enucleation. Those who undergo systemic chemotherapy, although there is tumor reduction, become more likely to develop other cancers. Multiple adverse effects are noted in those who receive intra-arterial delivery of the chemotherapeutics. Radiation therapy, similar to chemotherapy, has its adverse effects and lacks the ability to distinguish between healthy and cancerous cells. Targeted therapy has the potential to deliver more effective therapy, as it is designed to act on specific molecular targets, such as PDGFR-β, in a cell-specific fashion.

ACKNOWLEDGEMENTS

We would like to thank the Flow Cytometry and Cell Sorting Core facility, the Molecular Resource Center, and the members of the Morales-Tirado Laboratory. This work was funded by the Neuroscience Institute (ZKG), the Gerwin Fellowship (ZKG, VMT), St. Baldrick's Foundation (ZKG), Fight for Sight (ZKG), SJCRH Chair Endowment (MWW), The Ocular Oncology Fund (VMT, MWW), 930 Friends (VMT), and Research to Prevent Blindness (PI: JCF).

AUTHOR'S CONTRIBUTIONS

Conception and design of the work: MWW, and VMT; data acquisition, analysis, and interpretation: ZKG, ASI, MWW, and VMT; manuscript draft and significant contribution in intellectual content: ZKG, ASI, MWW, and VMT. All authors approved final version of manuscript. The following authors have no competing interests: ZKG, ASI, MWW, and VMT.

DATA AVAILABILITY STATEMENT

The chemical structure and compound summary of imatinibmesylate are available in PubChem, https://pubchem.ncbi.nlm.nih.gov CIDs: 123596.

All cell lines utilized in this investigation are commercially available: Primary human retinal microvascular endothelial cells are available from Cell Systems, https://cell-systems.com Cat. # ACBRI 181; Y79 Rb cells are available at the American Type Culture Collection (ATCC), www.atcc.org Cat. # HTB-18.

ETHICS APPROVAL AND CONSENT

This study did not require approval by The University of Tennessee Health Science Center Institutional Animal Care and Utilization Committee (IACUC) and Institutional Review Board (IRB).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Rodriguez-Galindo C, Orbach DB, VanderVeen D. Retinoblastoma. *Pediatr Clin North Am*. 2015; 62(1): 201-223. doi: 10.1016/j.pcl.2014.09.014

2. Goddard AG, Kingston JE, Hungerford JL. Delay in diagnosis of retinoblastoma: risk factors and treatment outcome. *Br J Ophthalmol*. 1999; 83(12): 1320-1323. doi: 10.1136/bjo.83.12.1320

3. Rodriguez-Galindo C, Chantada GL, Haik BG, Wilson MW. Treatment of retinoblastoma: Current status and future perspectives. *Curr Treat Options Neurol*. 2007; 9(4): 294-307. doi: 10.1007/s11940-007-0015-4

4. Balmer A, Zografos L, Munier F. Diagnosis and current management of retinoblastoma. *Oncogene*. 2006; 25(38): 5341-5349. doi: 10.1038/sj.onc.1209622

5. Goldsmith ZK, Coppess W, Irvine AS, Yuan K, Barsh SR, Ritter MK, et al. Targeting the platelet-derived growth factor-beta stimulatory circuitry to control retinoblastoma seeds. *Invest Ophthalmol Vis Sci*. 2018; 59(11): 4486-4495. doi: 10.1167/iovs.18-24359

6. Apte RS, Harbour JW. Inhibiting angiogenesis in retinoblastoma. *Ophthalmic Res* 2007; 39(4): 188-190. doi: 10.1159/000103578

7. Lee SY, Kim DK, Cho JH, Koh JY, Yoon YH. Inhibitory effect of bevacizumab on the angiogenesis and growth of retinoblastoma. *Arch Ophthalmol*. 2008; 126(7): 953-958. doi: 10.1001/archopht.126.7.953

8. Ravandi F. Managing Philadelphia chromosome-positive acute lymphoblastic leukemia: Role of tyrosine kinase inhibitors. *Clin Lymphoma Myeloma Leuk*. 2011; 11(2): 198-203. doi: 10.1016/j.clml.2011.03.002

9. Steinle JJ, Zhang Q, Thompson KE, Toutounchian J, Yates CR, Soderland C, et al. Intra-ophthalmic artery chemotherapy triggers vascular toxicity through endothelial cell inflammation and leukostasis. *Invest Ophthalmol Vis Sci*. 2012; 53(4): 2439-2445. doi: 10.1167/iovs.12-9466

10. Zhang Q, Jiang Y, Toutounchian J, Wilson MW, Morales-Tirado V, Miller DD, et al. Novel quinic acid derivative KZ-41 prevents retinal endothelial cell apoptosis without inhibiting retinoblastoma cell death through p38 signaling. *Invest Ophthalmol Vis Sci*. 2013; 54(9): 5937-5943. doi: 10.1167/iovs.13-12326

11. Reid TW, Albert DM, Rabson AS, Russell P, Craft J, Chu EW, et al. Characteristics of an established cell line of retinoblastoma. *JNCI Journal of the National Cancer Institute*. 1974; 53(2): 347-360. doi: 10.1093/jnci/53.2.347

12. Webb AH, Gao BT, Goldsmith ZK, Irvine AS, Saleh N, Lee RP, et al. Inhibition of MMP-2 and MMP-9 decreases cellular migration, and angiogenesis in in vitro models of retinoblastoma. *BMC Cancer*. 2017; 17(1): 434. doi: 10.1186/s12885-017-3418-y

13. Chintalapudi SR, Djenderedjian L, Stiemke AB, Steinle JJ, Jablonski MM, Morales-Tirado VM. Isolation and molecular profiling of primary mouse retinal ganglion cells: Comparison of phenotypes from healthy and glaucomatous retinas. *Front Aging Neurosci*. 2016; 8: 93. doi: 10.3389/fnagi.2016.00093

14. Abramson DH, Dunkel IJ, Brodie SE, Marr B, Gobin YP. Superselective ophthalmic artery chemotherapy as primary treatment for retinoblastoma (chemosurgery). *Ophthalmology*. 2010; 117(8): 1623-1629. doi: 10.1016/j.ophtha.2009.12.030

15. Ghassemi F, Shields CL. Intravitreal melphalan for refractory or recurrent vitreous seeding from retinoblastoma. *Arch Ophthalmol*. 2012; 130(10): 1268-1271. doi: 10.1001/archophthalmol.2012.1983

16. Schmack I, Hubbard GB, Kang SJ, Aaberg TM, Jr., Grossniklaus HE. Ischemic necrosis and atrophy of the optic nerve after periocular carboplatin injection for intraocular retinoblastoma. *Am J Ophthalmol*. 2006; 142(2): 310-315. doi: 10.1016/j.ajo.2006.02.044

17. Gobin YP, Dunkel IJ, Marr BP, Brodie SE, Abramson DH. Intra-arterial chemotherapy for the management of retinoblastoma: four-year experience. *Arch Ophthalmol*. 2011; 129(6): 732-737. doi: 10.1001/archophthalmol.2011.5

18. Wong JR, Morton LM, Tucker MA, Abramson DH, Seddon JM, Sampson JN, Kleinerman RA. Risk of subsequent malignant neoplasms in long-term hereditary retinoblastoma survivors after chemotherapy and radiotherapy. *J Clin Oncol*. 2014; 32(29): 3284-3290. doi: 10.1200/JCO.2013.54.7844

19. Daniels AB, Froehler MT, Pierce JM, Nunnally AH, Bozic I, Tao Y, et al. Efficacy, Toxicity and Biodistribution of Intra-arterial vs. Intravenous Chemotherapy in a Rabbit Retinoblastoma Model. In: Association for Research in Vision and Ophthalmology. Honolulu, HI; 2018.

20. Crane IJ, Liversidge J. Mechanisms of leukocyte migration across the blood–retina barrier. *Semin Immunopathol*. 2008; 30(2): 165-177. doi: 10.1007/s00281-008-0106-7

21. Xu H, Manivannan A, Goatman KA, Jiang HR, Liversidge J, Sharp PF, et al. Reduction in shear stress, activation of the endothelium, and leukocyte priming are all required for leukocyte passage across the blood--retina barrier. *J Leukoc Biol*. 2004; 75(2): 224-232. doi: 10.1189/jlb.1002479

22. Pennock S, Kim LA, Kazlauskas A. Vascular endothelial cell growth factor a acts via platelet-derived growth factor receptor alpha to promote viability of cells enduring hypoxia. *Mol Cell Biol*. 2016; 36(18): 2314-2327. doi: 10.1128/MCB.01019-15

23. Steinle JJ. Retinal endothelial cell apoptosis. *Apoptosis*. 2012; 17(12): 1258-1260. doi: 10.1007/s10495-012-0777-3

24. Kang SJ, Grossniklaus HE. Rabbit model of retinoblastoma. *Journal of Biomedicine and Biotechnology*. 2011; 2011: 394730. doi: 10.1155/2011/394730