

**Efficacy of Topical Delivery of Potentially Therapeutic Peptides and
Monoclonal Antibodies to the Posterior Segment of the Rat Eye in Eye
Drops versus PharmaLight Technology**

A thesis presented to the graduate faculty of The New England College of Optometry in
partial fulfillment of the requirements for the degree of Master of Science

Candice Ann Robinson

May 2008

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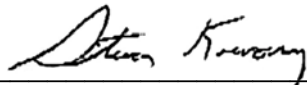
The author does not have any proprietary interest in the PharmaLight Nebulizer used in this study. The thesis advisor of this project, Dr. Steve Koevary, does have proprietary interest in the PharmaLight Nebulizer.

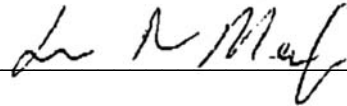
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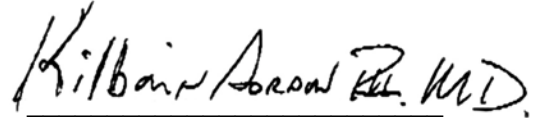
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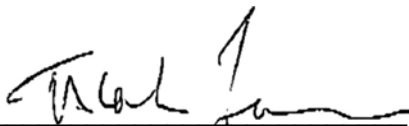
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Abstract

Efficacy of Topical Delivery of Potentially Therapeutic Peptides and Monoclonal Antibodies to the Posterior Segment of the Rat Eye in Eye Drops versus PharmaLight Technology

Candice A. Robinson

The New England College of Optometry, May 2008

Purpose

The PharmaLight Device is a new non-invasive topical delivery technology aimed at delivering pharmaceuticals to the ocular posterior segment efficiently without the risk of systemic side effects. This study seeks to determine the feasibility of delivering two separate large molecular weight peptides to the retina, sclera, optic nerve, and CSF using the PharmaLight device in comparison to standard eye drop delivery. Specifically, we examined the ability of the device to deliver leptin, a 16 KDa peptide, and IgG1, an antibody molecule with a molecular weight of 165 KDa.

Methods

An ELISA assay was used to determine the leptin and mouse IgG1 levels in the retina, sclera, optic nerve, cerebral spinal fluid (CSF) and serum post topical delivery with the PharmaLight device and standard eye drop delivery in rats.

Results

The use of the PharmaLight device with or without the permeation enhancer, 1% saponin, did not result in increased serum levels of leptin. Conversely, eye drop

delivered leptin without saponin resulted in increased serum leptin levels in comparison to baseline ($p < 0.01$). Significant levels of leptin in CSF was measured at 10 minutes with PharmaLight delivery of leptin with the use of saponin, but not with eye drop delivery of leptin with or without saponin ($p < 0.05$). Increased leptin levels were seen in the aqueous humor with eye drop delivery ($p < 0.01$) but not with device delivery of leptin. Retinal levels of leptin were increased after 20 minutes with both eye drop delivery and device delivery of leptin with or without the presence of saponin ($p < 0.01$). A continuous two minute device application of leptin resulted in a significant increase in leptin levels in the optic nerve and scleral tissues ($p < 0.05$). Mouse IgG1 levels were significantly increased in the optic nerve 10 minutes after a continuous five minute device application time ($p < 0.05$).

Conclusion

This data suggests that the PharmaLight device may prove to be an efficient, safe, and effective method for the delivery of large molecular weight peptides and monoclonal antibodies to the posterior segment for the treatment of ocular disease.

Table of Contents

Introduction.....	1
PharmaLight device technology.....	4
Potential use of PharmaLight for delivery to posterior segment...	5
Study Objective.....	10
Design and Methods.....	11
Animals.....	11
Verification of steady stream of mist.....	11
Uptake of leptin in tissues using PharmaLight.....	12
Effects of application time on leptin uptake.....	14
Uptake of IgG1 in optic nerve and retina with PharmaLight.....	15
Statistical analyses.....	16
Results.....	17
Experiment 1: Serum levels of leptin with drop vs. device.....	18
Experiment 2: CSF levels of leptin with drop vs. device.....	21
Experiment 3: Aqueous humor levels of leptin with drop vs device.	23
Experiment 4: Retina leptin levels of leptin with drop vs device...	24
Experiment 5: Effects of application time with device on tissues...	26
Experiment 6: IgG1 levels in optic nerve and serum with device...	30
Discussion.....	33
Conclusion.....	37
References.....	38

List of Figures and Tables

Figure 1.	PharmaLight Device Adapted for Rat Use	17
Figure 2.	Serum Leptin Levels After Topical Application - with Saponin	18
Figure 3.	Serum Leptin Levels After Topical Application - without Saponin	19
Figure 4.	Effects of Leaving a Drop of Leptin on the Eye for a Full 10 min or Misting the Eye with Leptin for a Full 10 min on Serum Leptin Levels	20
Figure 5.	CSF Leptin Levels After Topical Application with Saponin	21
Figure 6.	CSF Leptin Levels After Topical Application - without Saponin	22
Figure 7.	Aqueous Humor Leptin Levels After Topical Application – no saponin	23
Figure 8.	Retina Leptin Levels After Topical Application - with Saponin	24
Figure 9.	Retina Leptin Levels After Topical Application - without Saponin	25
Table 1.	Effects of Application Time on Leptin Uptake Into the Optic Nerve	26
Figure 10.	Effects of Leptin Application Time Using the Device on Scleral Uptake	27
Figure 11.	Leptin Levels in the Sclera of Rats Sacrificed 10 Min After Application of a Continuous 10 min Leptin Mist	28
Figure 12.	Leptin Levels in the Retina of Rats Sacrificed 10 Min After Application of a Continuous 10 min Leptin Mist	29
Figure 13.	Mouse IgG1 Levels in Optic Nerves at 2, 5, 10 Min with Device	30
Figure 14.	Serum IgG1 levels following topical application with the PharmaLight device	31

INTRODUCTION

The eye presents unique challenges when it comes to the topical delivery of pharmaceuticals (Geroski & Edelhauser, 2000; Davies, 2000). Some of these challenges stem simply from the diversity of the ocular tissue compartments, each with its own permeability and solubility characteristics. Additionally, topically applied drugs are subject to rapid drug elimination due to lacrimal and nasolacrimal drainage, drug binding to and metabolism by tear proteins, and target non-specificity resulting from systemic absorption through the nasal and lacrimal duct mucosa and conjunctival vasculature (Stjernschantz & Astin, 1993; Patton & Francoeur, 1978; Frishman, Kowalski, and Nagnur, 2001). Despite these challenges, clear advantages do exist for drug administration by the topical route. Topical delivery offers a non-invasive, direct, localized delivery to the target tissue, better accessibility into the intraocular environment than can generally be achieved by systemic delivery, avoidance of hepatic first-pass metabolism, and convenience (Koevary, Lam, and Patsiopoulos, 2004).

To date, there are no first line topical pharmaceuticals for the treatment of many diseases of the ocular posterior segment. However, research over the years has suggested that posterior segment diseases such as diabetic retinopathy, age-related macular degeneration (AMD), idiopathic uveitis, and retinitis pigmentosa might be treatable with topically applied drugs. Data to support the notion that topically applied drugs can reach the back of the eye include: 1. topical anti-glaucoma medications influence retinal blood flow (Arend, Harris, and Arend, 1998; Grunwald 1990; Haefliger, Lietz, and Griesser, 1999); 2. topical nepafenac, a non-steroidal, anti-inflammatory prodrug which inhibits choroidal neovascularization and ischemia-induced retinal neovascularization by

decreasing the production of VEGF (Takahashi, Saishin, and Mori K, 2003); and 3. very low, but potentially therapeutic, concentrations of the aldose reductase inhibitor, imirestat, and dexamethasone accumulate in the vitreous following topical application (Maurice, 2002). It is important to note that all of the above compounds have a low molecular weight. In addition to these examples, our previous work showed that the peptide insulin could accumulate in the retina, optic nerve, cerebrospinal fluid (CSF), and distinct regions of the central nervous system (CNS) following topical application (Koevary, Nussey, and Lake, 2002; Koevary, 2003; Patsiopoulos, Lam, and Lake, Koevary, 2003).

There are two general pathways that a drug can take from the ocular surface to the posterior segment: 1. Corneal – through the anterior chamber, lens, pupil, or the iris or its root; and 2. Conjunctival – directly across the sclera, choroid, choriocapillaris, and retinal pigment epithelium (RPE), or indirectly into the retrobulbar space and the optic nerve head (Maurice, 2002). It has been estimated that the maximum concentration of drug in the aqueous humor occurs between 0.5 and 3 hours following topical application and the concentration is more diluted for a hydrophilic drug such as fluorescein and less diluted for a lipophilic drug like the β -blocker, metipranolol (Maurice, 2002; Kessler, Bleckmann, and Kleintges, 1991; Joshi, Maurice, and Paugh, 1996). Once in the aqueous humor, drugs would have ready access to the posterior surface of the iris, which was estimated to have a permeability greater than the conjunctival and corneal epithelium but somewhat less than the RPE (Joshi, Maurice, and Paugh, 1996; Macdonald & Maurice, 1991). High molecular weight drug delivery to the posterior sclera has been thought to occur by transport through the suprachoroidal space, while smaller molecular weight

drugs have been proposed to gain entry into the vitreous near the pars plana. It has also been suggested that with conjunctival delivery, drugs may permeate into and gain access to the posterior sclera and orbit (Hosoya, Lee, and Kim, 2005).

Eye drops containing ocular drug formulations typically give rise to high and unpredictable initial concentrations on the ocular surface, followed by a rapid decline to very low levels. This pattern of drug delivery has been referred to as pulse entry, which approximates first-order kinetics and necessitates the frequent administration of drug in order to maintain the ocular concentration above the minimally effective dosage. However, to account for these first order kinetics, drug solutions have to be administered at high concentrations which may consequently trigger systemic side effects (Bourgeois, 1991; Rait, 1999). While methods that prolong ocular residence time and enhance corneal permeability have been developed, results have been variable and, in many instances, drug specific (Mainardes, Urban, and Cinto, 2005). The use of biocompatible polymers has also been shown to be somewhat effective in delivering drugs to the posterior segment (Alonso & Sanchez, 2003; Frangie, 1995; Ludwig, Van Haeringen, and Bodelier, 1992); however, given the diversity of polymer-based drug delivery systems, it is unrealistic to expect that a single universal polymer will be able to be used in all ocular delivery systems.

On the other hand, effective delivery of a host of diverse drugs to the posterior segment may be achievable using the PharmaLight device. The use of this device could be potentially most useful in delivering therapeutic proteins and peptides to the retina and optic nerve. Delivery of proteins and peptides is most challenging because these molecules are generally of high molecular weight, hydrophilic, and susceptible to

degradation by peptidases in the eye, and they pose other difficulties with respect to their formulation into viable dosage forms. The rapid delivery of drugs through the eye with this device could theoretically minimize the negative effects of metabolic enzymes that are found in many ocular tissues. As previously mentioned, drugs that are applied in high concentrations to the ocular surface would have access to pathways through the eye that would allow them to diffuse into the posterior segment. Theoretically, these pathways would similarly facilitate the transport of drugs that are applied with the PharmaLight device.

PharmaLight Device Technology:

PharmaLight's innovative ophthalmic drug delivery system comprises an ophthalmic drug device and proprietary formulation that may enable the safe, effective, and non-invasive administration of therapeutics, including large molecules, to the posterior segment.

The drug device portion (see figure at beginning of Results section) of the ophthalmic drug delivery system is composed of a misting unit that includes a nebulizer that is configured to generate mist from a pharmaceutical composition, and a mist director to direct the mist to the eye. The nebulizer contains a piezoelectric crystal which, when activated, vibrates rapidly to generate the pharmaceutical mist from the drug reservoir. The nebulizer is adapted for variable frequency in order to vary the mean diameter of the droplets of mist of $\leq 3-10$ microns, a size that doesn't trigger blinking or

tearing even when irritating enhancers are used. This size distribution provides for a uniform spread of droplets and a greater coverage of the target tissues.

Potential use of the PharmaLight device to deliver angiogenesis inhibitors to the posterior segment for the treatment of age-related macular degeneration (AMD):

The focus of this project was to examine whether the PharmaLight device can deliver relatively large molecular peptides to the posterior segment of the eye. Potentially therapeutic peptides for the treatment of posterior conditions will likely be larger, neurotrophic factors and antibody molecules. Neovascular retinal conditions such as diabetic retinopathy and AMD are leading causes of vision loss worldwide (van Wijngaarden, Coster, and Williams, 2005; Congdon, O'Colmain, and Klaver, 2004; Kempen, O'Colmain, Leske, 2004). Central to the pathogenesis of neovascularization is increased vascular permeability leading to retinal edema, subretinal fluid accumulation, and the proliferation of new vessels that are prone to hemorrhage (van Wijngaarden et al., 2005). The established therapy for retinal neovascularization in diabetic retinopathy i.e., laser photocoagulation, may be effective in delaying the progression of the disease but it lacks specificity and is associated with retinal destruction, resulting in impaired visual function (Kaufman, Ferris, and Seigel, 1989). The management of choroidal neovascularization in AMD has been bolstered by the advent of photodynamic therapy (PDT); however it is only helpful in a small subset of neovascular lesions. Additionally, while PDT is often effective in ablating established pathological vessels, it does not

prevent new vessel formation, and repeated treatments are often required (No authors listed, 1999).

In light of the above, it is clear that there is a need for treatments that selectively target the molecular mediators of ocular neovascularization. One such approach involves the use of specific monoclonal antibodies. While relevant targets for antibodies that have been under investigation include those directed against transforming growth factor beta (TGF- β) (Triolo, Vadala, and Accardo-Palumbo, 2002) and connective tissue growth factor (CTGF), most have focused on vascular endothelial growth factor (VEGF). The VEGF family comprises six secreted glycoproteins that are designated VEGF A through E, and placental growth factor (Ferrara, Gerber, and LeCouter, 2003; Houck, 1991; Tischer, Mitchell, and Hartman, Silva, 1991). The best characterized of these is VEGF-A (commonly referred to simply as VEGF and also known as vascular permeability factor), which is a 34- to 45-kD homodimeric glycoprotein. While VEGF plays an important role in such diverse processes as wound healing, ovulation, menstruation, maintenance of blood pressure, and pregnancy, it also plays a pivotal role in the development of pathological angiogenesis in ischemic and inflammatory disease including arthritis, psoriasis, macular degeneration, and diabetic retinopathy. VEGF has been identified in neovascular membranes in both diabetic retinopathy and AMD (Kvanta, Alverre, and Berglin, 1996; Aiello, Avery, and Arrigg, 1994) and elevations in VEGF levels were reported in the aqueous humor and vitreous humor of human eyes with neovascular conditions (Aiello, Avery, and Arrigg, 1994; Malecaze, Clemens, and Simorer-Pinotel, 1994). As confirmed in animal models, these studies demonstrated a temporal correlation between VEGF elevations and active proliferative retinopathy.

Subsequently, animal studies using various VEGF inhibitors including soluble VEGF receptor chimeric proteins (Aiello, Pierce, and Foley , 1995), monoclonal antibodies (Adamis, Shima, and Tolentino, 1996), antisense oligonucleotides (Robinson, Pierce, and Rook,1996), and small molecule VEGFR-2 kinase inhibitors (Ozaki, Seo, and Ozaki, 2000), directly confirmed the role of VEGF as a key mediator of ischemia-induced intraocular neovascularization.

Neovascularization, specifically the development of choroidal neovascular membranes, and vascular leakage are major causes of visual loss in the wet form of AMD (Garner A, 1994). As mentioned above, VEGF has been identified in neovascular membranes in AMD patients, suggesting a role for VEGF in the progression of AMD-related choroidal neovascularization. Over the past few years, the effects of several anti-VEGF agents were explored in AMD patients including a recombinant humanized anti-VEGF Fab (Lucentis-Genentech), a recombinant humanized full length antibody to VEGF (Avastin, currently FDA approved for the treatment of colorectal cancer-Genentech), and a 2'-fluoropyrimidine RNA oligonucleotide ligand (aptamer; Macugen-Eyetech). Macugen binds only to VEGF-A while Avastin and Lucentis bind to all VEGF isoforms. Studies have shown that the vision of patients treated with Macugen continued to worsen during the course of treatment, perhaps because this agent only targets a single isoform of VEGF (Kourlas & Schiller, 2006). A small number of patients injected intravenously with Avastin showed significant improvement in their visual acuity at 3 months and in central retinal thickness as early as 1 week post-infusion (Michels, Rosenfeld, and Puliafito, 2005). There remained a concern, however, about the elevated risk of thromboembolic events in these patients. Pieramici reported that the intravitreal

injection of Avastin in AMD patients resulted in the transient resolution of retinal edema (Pieramici, Avery, and Rabena, 2006). A few years ago, Lucentis was reported to reduce angiogenesis and vascular leakage in a primate model of AMD (Krzystolik, Afshari, and Adamis, 2002), which led to human studies, most notably the MARINA (Minimally classic occult trial of the Anti-VEGF antibody Ranibizumab In the treatment of Neovascular AMD) study. The MARINA study is a Phase III study of 716 patients in the US with minimally classic or occult wet AMD who were randomized to receive intravitreal Lucentis injections or a control regimen. In June of 2006, the FDA approved Lucentis (0.5 mg intravitreal injection) for the treatment of wet AMD. A recent study conducted by Regillo 2008, showed that Lucentis significantly arrested the growth and reduced the leakage of choroidal neovascular membranes associated with wet AMD. Additionally, Lucentis was shown to significantly improve visual acuity in patients treated monthly for three consecutive months (Regillo, Brown, and Abraham, 2008).

While both Avastin and Lucentis appear to be effective in not only preventing vision loss in AMD but also reversing it, a major concern of both of these treatments is the need for repeated, invasive, intravitreal injections, and its associated risks that include endophthalmitis and retinal detachment (Gragoudas, Adamis, and Cunningham, 2004). Furthermore, while VEGF antagonists appear to be well tolerated in the short term, a growing body of evidence from animal and in vitro experiments and from the experience with Avastin in colorectal cancer patients, hints at the potential for serious systemic adverse effects. Preclinical studies of a recombinant humanized whole antibody closely related to Lucentis in young adult cynomolgus monkeys revealed it's potential to induce physeal dysplasia following biweekly intravenous injections of low doses (Ryan, Eppler,

Hagler, 1999). VEGF plays other vital roles, such as the formation of collateral vessels critical to the viability of the myocardium. Thus, since individuals with diabetic retinopathy and AMD may be at increased risk of cardiovascular and peripheral vascular disease, the implications of long-term systemic inhibition of VEGF could be profound (Snow & Seddon, 1999; Klein, Klein, and McBride, 2004; Csaky, 2003). For these reasons, it is imperative that an alternative means of drug delivery be developed that can transport these agents to their target region at the back of the eye without resulting in significant systemic delivery.

Study Objective:

To examine the feasibility of delivering two fairly large molecular weight peptides to the retina, sclera, optic nerve, and CSF using the PharmaLight device. Specifically, we examined the ability of the device to deliver leptin, a 16KDa peptide, and IgG1, an antibody molecule with a molecular weight of 165KDa. The ability to deliver these peptides non-invasively to these tissues would bode well for the future use of this device for the delivery of potentially therapeutic peptides and antibodies to the posterior segment and beyond.

MATERIALS AND METHODS

Animals:

Female Lewis rats approximately 6-10 weeks of age were used for this study. The rats were obtained from colony 202B at Harlan (Indianapolis, IN) and were housed and maintained at New England College of Optometry's animal facility in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All experimental procedures were approved by the school's Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Verification that the device produced a steady stream of mist:

In an initial study, the device was loaded with 2 mL of phosphate buffered saline (PBS) buffer and was left on for one full minute; this was repeated 4 times and the amount of buffer that remained in the reservoir was determined using a P1000 pipettor. This procedure was performed in order to confirm that the device was delivering a uniform volume of mist per minute over a five minute period. In a second study, the device was used to deliver PBS mist to the eye of an anesthetized rat and the above procedure was repeated such that the volume of mist that was absorbed into the eye was calculated over a 5 minute period. In a final study, the latter study was repeated using buffer that contained 1% saponin, the permeation enhancer that we planned to use in subsequent experiments.

Uptake of leptin in the retina, serum, aqueous humor, and CSF using the device with and without 1% saponin:

Studies were undertaken to assess the capacity of the PharmaLight device to deliver a relatively large molecular weight peptide i.e., leptin (16 kDa) into the retina, optic nerve, sclera, serum, and CSF; this peptide is a non-glycosylated molecule (56). The CSF was examined because it envelopes the optic nerve; we previously showed that topically applied insulin that accumulated in the optic nerve also accumulated in the CSF and certain brain regions. We chose to examine the CSF because we were interested in determining the potential the device has for delivering peptides into the CNS.

Cohorts of Lewis female rats (n=3-11/group) were treated with a 600 µg/ml solution of leptin in the absence or presence of the permeation enhancer saponin (1%). Leptin was applied with the device for two minutes and the amount of leptin actually delivered was determined by averaging the amount missing from the device after every four rats were misted. In some instances, however, shorter or longer mist times were used. When rat eyes were misted with the device for two minutes or less, conscious animals were used. In the experiments in which longer mist times were used i.e., 5, 10, or 20 minutes, the animals were first anesthetized with xylazine/ketamine (1.25/6.25 mg/100 gbw). Finally, an additional group of rats was treated with a 15 µL drop of leptin solution which served as a basis for comparison of the efficacy of the device relative to standard topical delivery. In all instances, the animal's left eye was the treated eye.

For tissue collection, the animals were sacrificed by decapitation at various time points post application and their serum, CSF, aqueous humor, retina, optic nerve, and/or sclera were harvested. Aqueous humor was removed using a 50 μ L Hamilton syringe with a 30G needle; on average, approximately 25-30 μ L of fluid was harvested per rat. In all experiments (including those described in the section below), aqueous humor was removed from all eyes prior to the harvesting of ocular tissues, regardless of whether the aqueous humor samples were analyzed by ELISA. This method of aqueous collection reduces the chances of tissue cross-contamination. The sclera were harvested by first cutting the globe at the equator and then scraping the retina free using forceps. The optic nerve samples were harvested through the intracranial route; the nerves were cut rostral to the chiasm and were then pulled back from the orbit through the orbital foramen. Efforts were made to select segments of the optic nerve that were of similar length

The time points were calculated based on the start time of treatment. The tissues were homogenized in 120 μ L of assay buffer that contained a standard cocktail of protease inhibitors on ice, and the homogenates were then centrifuged for two minutes in a Beckman microfuge. They were then assayed, along with the serum and CSF, for the presence of leptin using an ELISA kit (Assay Designs, Ann Arbor, MI) in conjunction with a BioTek EL-310 plate reader. In instances where fluid samples needed to be diluted, the same dilution factor was used across all samples. CSF samples were microfuged for 10 seconds while blood samples were allowed to clot on ice after which they were centrifuged for 10 minutes at 2200rpm at 4°C in a Sorvall RT, 6000B refrigerated centrifuge. The protease buffer that was used was the one recommended by technical support at Assay Designs. To harvest the CSF animals that had not already

been anesthetized were then anesthetized 5 minutes prior to CSF collection with a 27G cannula through the cisterna magnum; approximately 50-100 μ L of CSF was harvested per rat. Data are expressed as pg/ml for the serum and CSF data, and as pg/ μ g protein for the tissue results (the protein concentrations of the cell pellets were determined using the BioRad reagent); values are the means \pm SEM.

Effects of application time on leptin uptake into the retina, optic nerve and sclera:

Cohorts of rats (3-4/group) were treated with a 600 μ g/ml solution of leptin containing 1% saponin. Leptin was applied with the device for varying periods of time from 15 seconds to 10 minutes. As mentioned, when rat eyes were misted with the device for two minutes or less, conscious animals were used. However, in the experiments in which a longer mist time was used i.e., 10 minutes, the animals were first anesthetized as described previously. As above, in all instances, the animal's left eye was the treated eye.

The animals were sacrificed by decapitation 10 minutes after the start of leptin application and their retina, sclera and/or optic nerve were harvested. As above, the sclera were harvested by first cutting the globe at the equator and then scraping the retina free using forceps. The optic nerve samples were harvested through the intracranial route; the nerves were cut rostral to the chiasm and were then pulled back from the orbit through the orbital foramen. As above, efforts were made to select segments of the optic nerve that were of similar length. The tissue samples were homogenized in 120 μ L of assay buffer that contained a standard cocktail of protease inhibitors on ice, and the

homogenates, following centrifugation for two minutes in a Beckman microfuge, were assayed, along with the serum and CSF, for the presence of leptin using the above ELISA and plate reader. Data are expressed as pg/ μ g protein and the values are the means \pm SEM.

Uptake of IgG1 in the optic nerve and retina following its application with the device in a formulation containing 1% saponin:

Cohorts of rats (2-5/group) were exposed to device-delivered mist containing mouse 1.6 μ g/mL IgG1, a molecule of 165kDa molecular weight, for either 2, 5, or 10 minutes. The IgG1 used in these experiments exhibits <1% cross reactivity with rat IgG1. In the interest of uniformity, all rats were anesthetized prior to treatment, as described above. Most animals were sacrificed 10 minutes after the start of treatment though a few that had their eyes misted for a full 10 minutes were sacrificed at 20 minutes. All animals had their optic nerves and serum harvested and prepared as previously described. Tissue homogenates were assayed using an IgG1 ELISA kit (Assay Designs). As above, efforts were made to select segments of the optic nerve that were of similar length; retina sizes have generally been similar between animals. Data were expressed as ng/ml. Admittedly, since neither tissue weights nor tissue protein levels were determined, our data can only be viewed as approximate.

Statistical Analyses:

Group means were compared using a Bonferroni corrected Student's t test.

RESULTS

Verification that the device produced a steady stream of mist:

The average volume of mist that was liberated into the air was 321.2 ± 24.4 $\mu\text{L}/\text{minute}$. The average volume of mist that was delivered into the eye over 5 minutes averaged 16.4 ± 2.3 $\mu\text{L}/\text{minute}$. The total amount of mist that entered the eye when saponin-containing buffer was used was 18 ± 1.9 $\mu\text{L}/\text{minute}$, which was not significantly greater than the non-saponin-containing mist. A picture of the PharmaLight device adapted for the rat is shown below in **Figure 1**.



Uptake of leptin in the serum, CSF, aqueous humor, and retina with and without 1% saponin:

Serum leptin levels following eye drop and device application:

Figure 2 below shows the serum leptin levels 10 and 20 minutes after rats received either a leptin drop or a two minute leptin mist in their left eye; in both cases, the formulation contained 1% saponin. Our data showed that following both the eye drop and mist application, serum leptin levels remained statistically unchanged, though their absolute levels did vary.

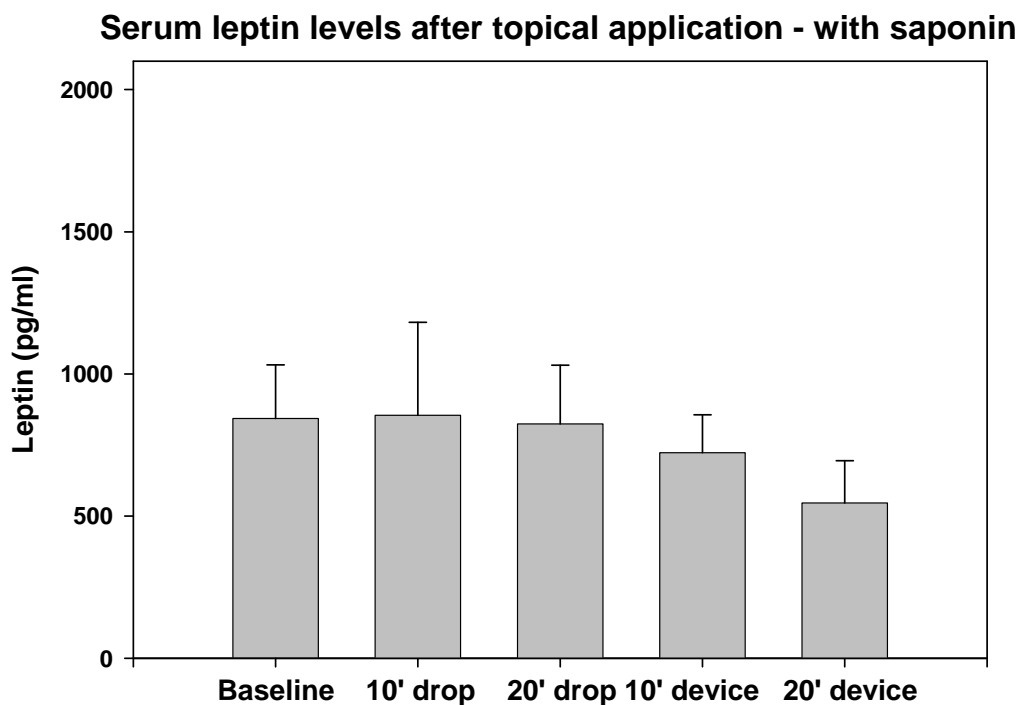
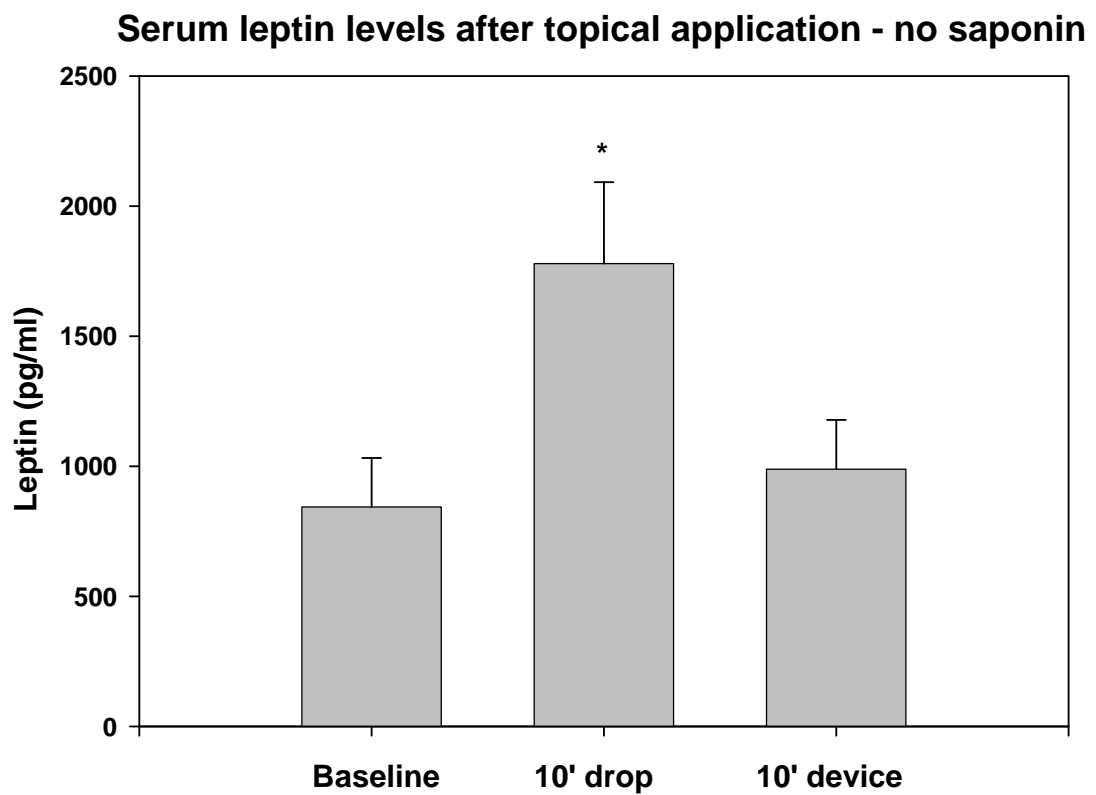


Figure 3.

	Baseline	10' drop	20' drop	10' device	20' device
Mean:	843.4329	854.8665	823.8907	722.4275	546.3775
SE:	188.2964	326.7486	206.4866	133.5824	148.1007
N:	13	4	11	4	4

Figure 3 below shows the serum leptin levels 10 minutes after rats received either a non-saponin containing leptin drop or a two minute leptin mist in their left eye; 20 minute points were not examined. Our data showed that while leptin levels rose significantly in the serum of eye drop treated rats, they remained unchanged in the mist treated rats.

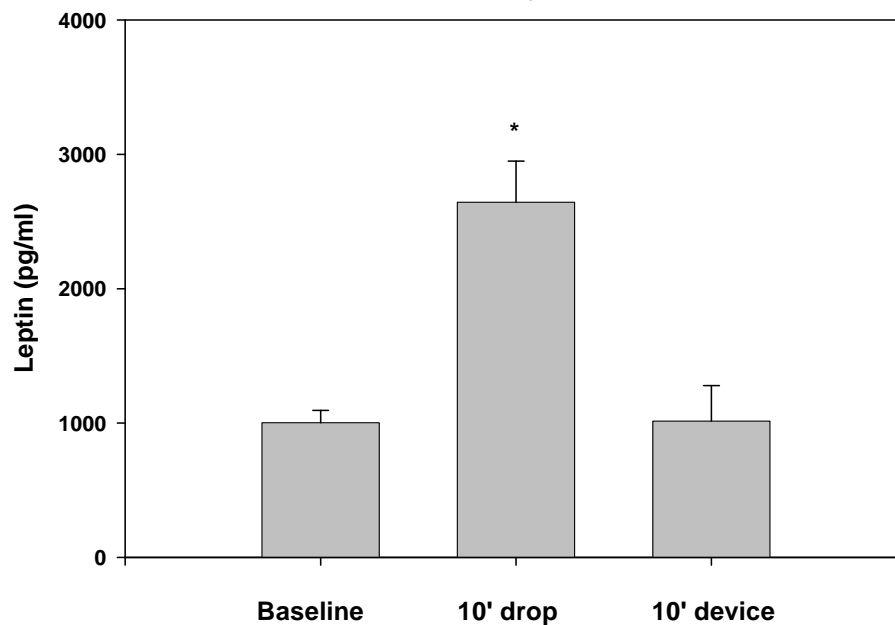


	Baseline	10' drop	10' device
Mean:	843.4329	1779.2415	988.6095
SE:	188.2964	312.2949	189.3462
N:	13	6	6

*significant vs. baseline, $p < 0.01$

The above data suggested that mist-applied leptin was not entering the blood. To further verify this, anesthetized rats were subjected to a leptin mist that contained saponin for 10 full minutes or had their eye treated such that a pool of leptin-containing buffer was visible on the eye for the full 10 minutes of application. The data, graphically shown below in **Figure 4**, revealed that under these circumstances, serum leptin levels rose after 10 minutes while serum leptin levels in device-treated rats did not change. This data is compelling in light of the above data showing that saponin-containing leptin drops did not induce an elevated in leptin levels when rats received a typical eye drop containing leptin.

Effects of leaving a drop of leptin on the eye for a full 10 min or misting the eye with leptin for a full 10 minutes on serum leptin levels

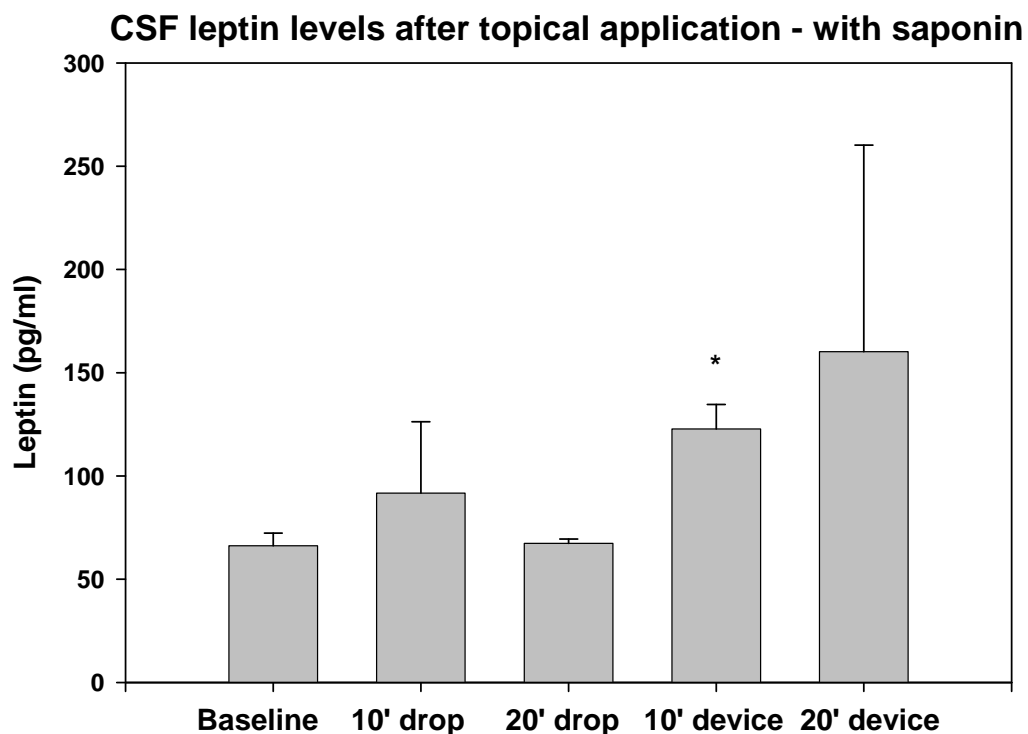


	Baseline	10' drop	10' device
Mean:	1001.5	2642.25	1294.75
SE:	92.34	306.88	167.92
N:	4	4	4

***significantly greater than baseline, $p < 0.004$**

CSF leptin levels following eye drop and device application:

Figure 5 below shows CSF leptin levels 10 and 20 minutes after rats received either a leptin drop or a two minute leptin mist in their left eye; in both cases, the formulation contained 1% saponin. Our data showed that CSF leptin levels were significantly elevated 10 minutes after leptin application with the device but not after eye drop application. Interestingly, serum leptin levels in these animals were unchanged relative to background, supporting the notion that elevations in CSF levels were due to direct transport from the front of the eye following mist application.

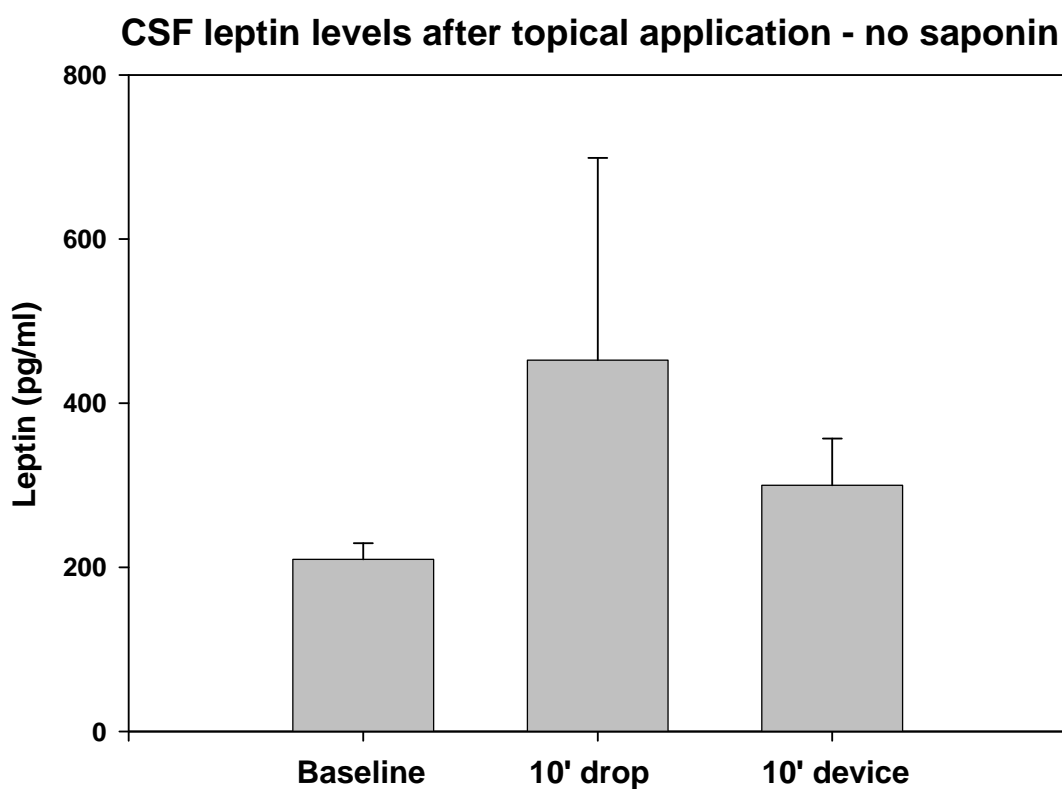


	Baseline	10' drop	20' drop	10' device	20' device
Mean:	66.2180	91.6624	67.3346	122.7740	160.1861
SE:	6.0468	34.5837	2.1721	11.8470	100.0029
N:	6	3	*2	10	4

(*lost sample)

***significant vs. baseline $p < 0.05$**

Figure 6 below shows CSF leptin levels 10 minutes after rats received either a non-saponin containing leptin drop or a two minute leptin mist in their left eye. Results showed that while mean CSF leptin levels were elevated 10 minutes after leptin application with the device or drops, these values did not reach statistical significance. In light of the above results, this data suggests that saponin may be required for CSF accumulation of leptin following mist application.

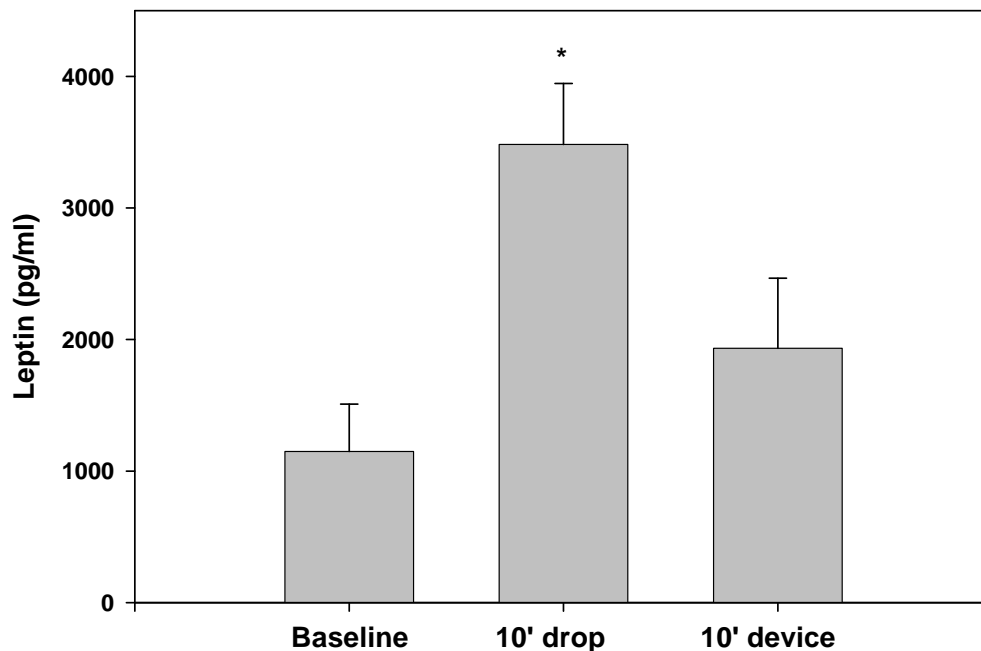


	Baseline	10' drop	10' device
Mean:	209.7620	452.3920	300.1047
SE:	19.5056	246.4289	56.9175
N:	3	3	3

Aqueous humor leptin levels following eye drop and device application:

The graph below shows aqueous humor leptin levels 10 minutes after rats received either a leptin drop or a two minute leptin mist in their left eye; in both cases, the formulation lacked saponin. Our data, shown in **Figure 7** below, showed that aqueous humor leptin levels were significantly elevated 10 minutes after leptin application with an eye drop, but not after leptin application with the device. At 10 minutes, serum leptin levels in these animals were unchanged relative to background, again supporting the notion that elevation in aqueous humor leptin levels were due to direct transport from the front of the eye following mist application (data not shown).

Aqueous humor leptin levels after topical application - no saponin

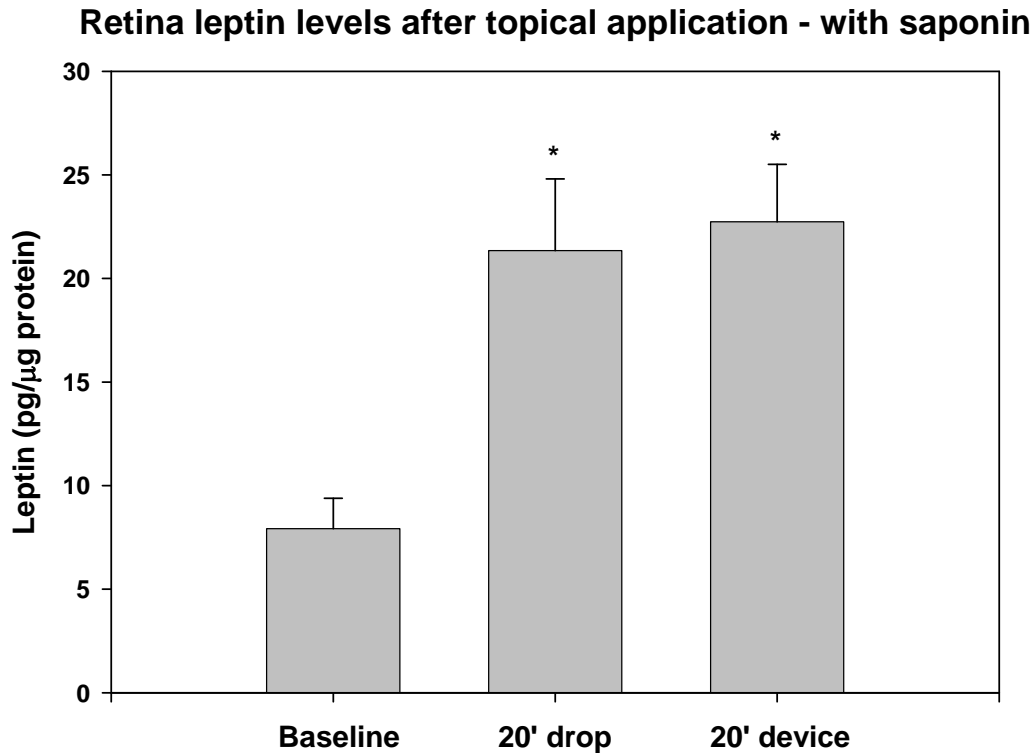


	Baseline	10' drop	10' device
Mean:	1148.4117	3483.0833	1933.2183
SE:	360.0465	463.4087	533.0370
N:	6	6	6

***significant vs. baseline, p<0.01**

Retina leptin levels following eye drop and device application:

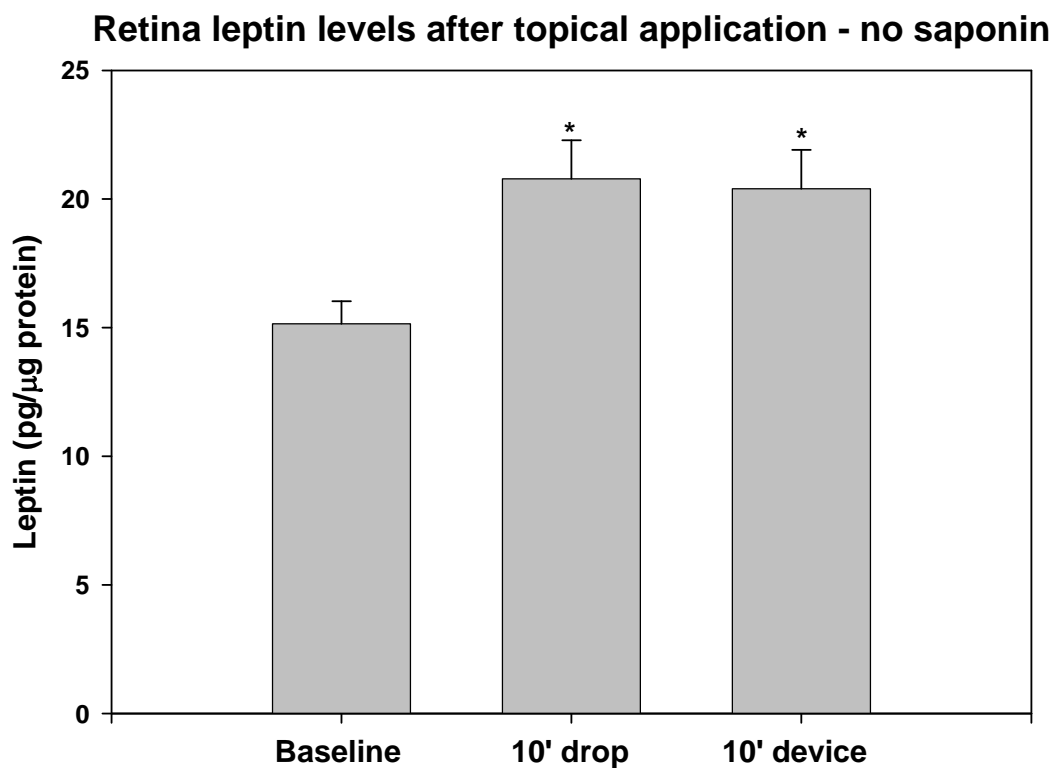
The data in **Figure 8** below shows retina leptin levels 20 minutes after rats received either a leptin drop or a 2 minute leptin mist in their left eye; in both cases, the formulation contained 1% saponin. Our data showed that retina leptin levels were significantly elevated 20 minutes after leptin application with either the device or eye drops. As above, it was interesting that serum leptin levels in these animals at these time points were unchanged relative to background, supporting the notion that elevations in retinal leptin levels were due to direct transport from the front of the eye following leptin application.



	Baseline	20' drop	20' device
Mean:	7.9209	21.3435	22.7363
SE:	1.4721	3.4600	2.7697
N:	4	4	3

***significant vs. baseline, p<0.01**

The data in **Figure 9** below shows retina leptin levels 10 minutes after rats received either a leptin drop or a two minute leptin mist in their left eye; in these cases, the formulation did not contain saponin. Interestingly, the data showed that in this case as in the data above in which saponin was used; retina leptin levels were significantly elevated 10 minutes after leptin application with either the device or eye drops. Again, the serum data support the notion that elevations in retinal leptin levels were due to direct transport from the front of the eye following leptin application (data not shown).



	Baseline	10' drop	10' device
Mean:	15.1454	20.7795	20.3982
SE:	8.5822	14.6669	14.7685
N:	3	3	3

***significant vs. baseline $p < 0.01$**

Note: Since endogenous leptin levels increase and then decrease after a meal, the somewhat different starting baseline values in the above experiments were likely a reflection of minor differences in the animals' feeding times during the previous night.

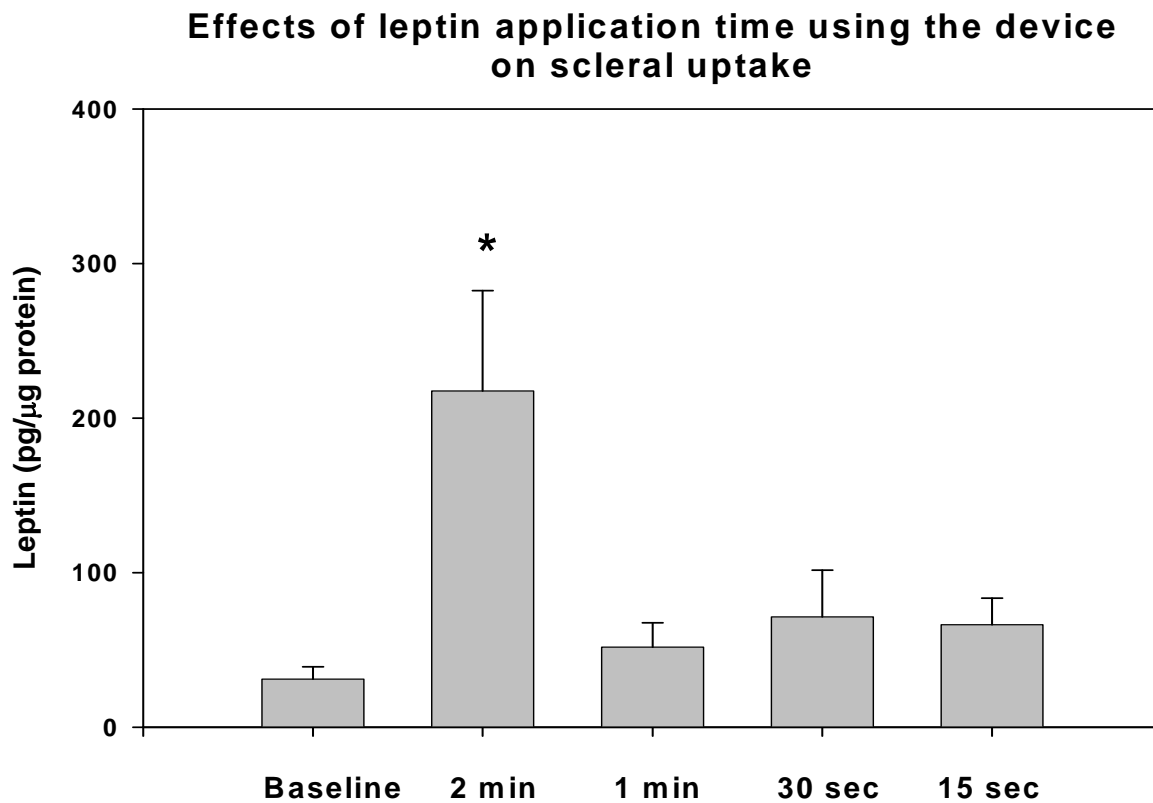
Effects of application time on leptin uptake into the retina, sclera, and optic nerve:

When leptin was applied to the rat eyes with the device for varying periods of time up to two minutes, only rats that had been treated for at least two full minutes exhibited leptin uptake in the optic nerve. Our results (pg/ μ g protein) are shown in Table 1 below. Low indicates that the values were below the detection limit of the ELISA.

Table 1. Optic nerve data

Baseline	2 min	1 min	30 sec	15 sec
Low	72\pm36 (mean\pmSEM)	Low	Low	Low

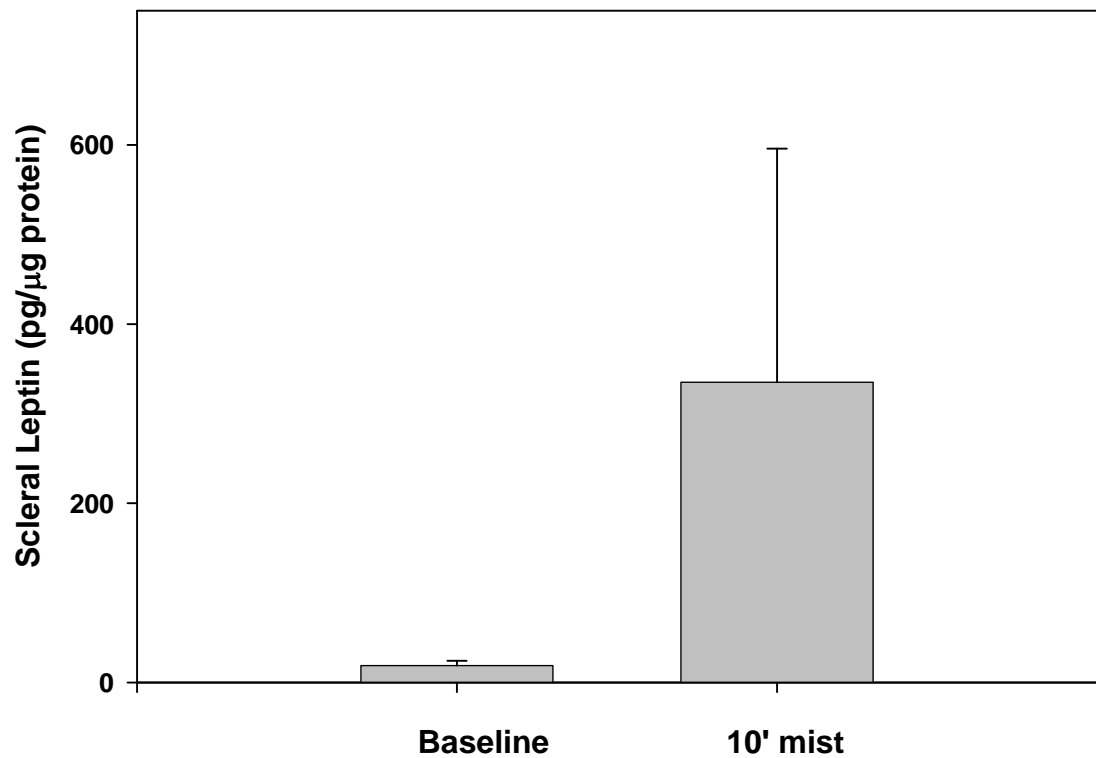
Our data were similar with regard to scleral uptake with only the two minute values showing significant elevations above baseline (see **Figure 10** below). It should be noted that in both the optic nerve and sclera, the concentration of leptin was markedly higher than in the retina.



*significantly higher than baseline, $p < 0.05$

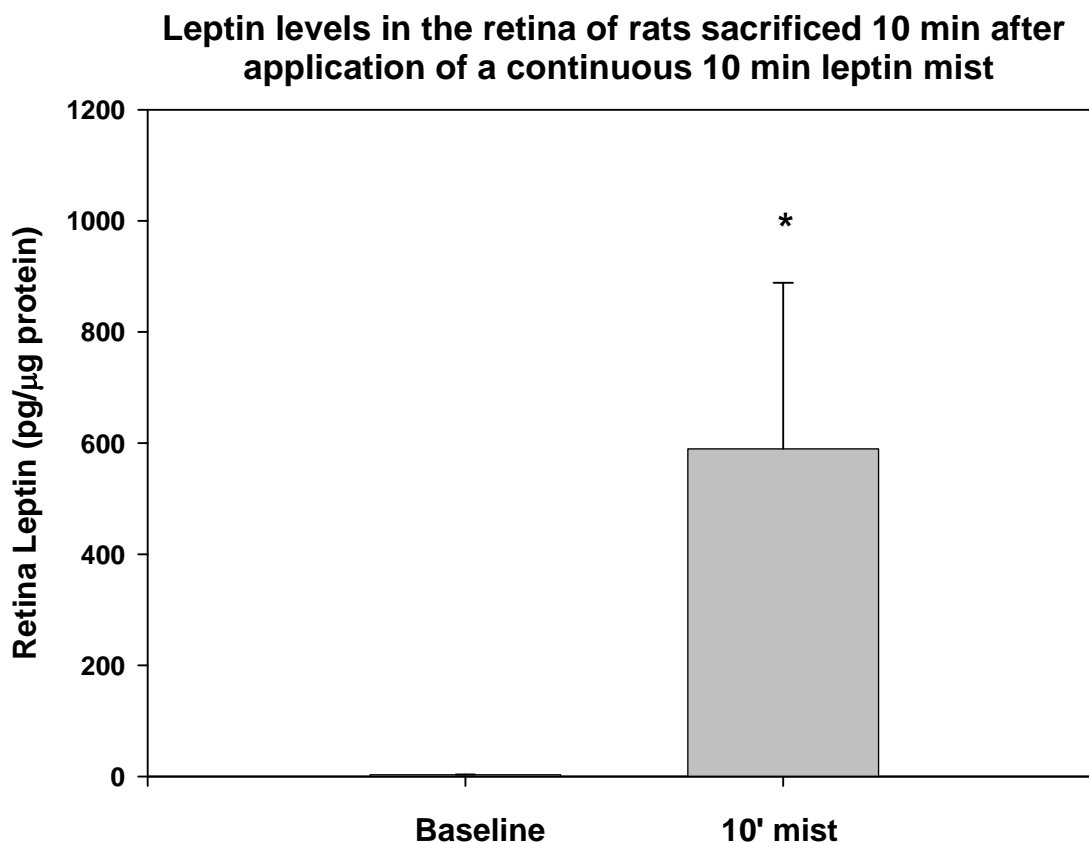
When rat eyes were exposed to device-delivered leptin for a full 10 minutes, levels in the sclera increased above levels seen with a two minute mist (above), though these differences were not statistically significant (see **Figure 11** below).

Leptin levels in the sclera of rats sacrificed 10 min after application of a continuous 10 min leptin mist



	Baseline	10' mist
Mean:	18.6565	334.9737
SE:	5.4348	260.8608
N:	3	4

When rat eyes were exposed to device-delivered leptin for a full 10 minutes (see **Figure 12** below), levels in the retina increased significantly ($p < 0.01$) above baseline and above the two minute values seen in animals sacrificed after 20 minutes (from data above; 23 ± 3 pg/ μ g protein). It should be noted, however, that there was no significant difference between retinal and scleral protein concentrations (26.6 ± 1.6 μ g and 28.4 ± 2.1 μ g respectively, $p = 0.5$) suggesting that the differences between the retina and sclera cannot be attributed to protein concentrations in the tissues (data not shown).



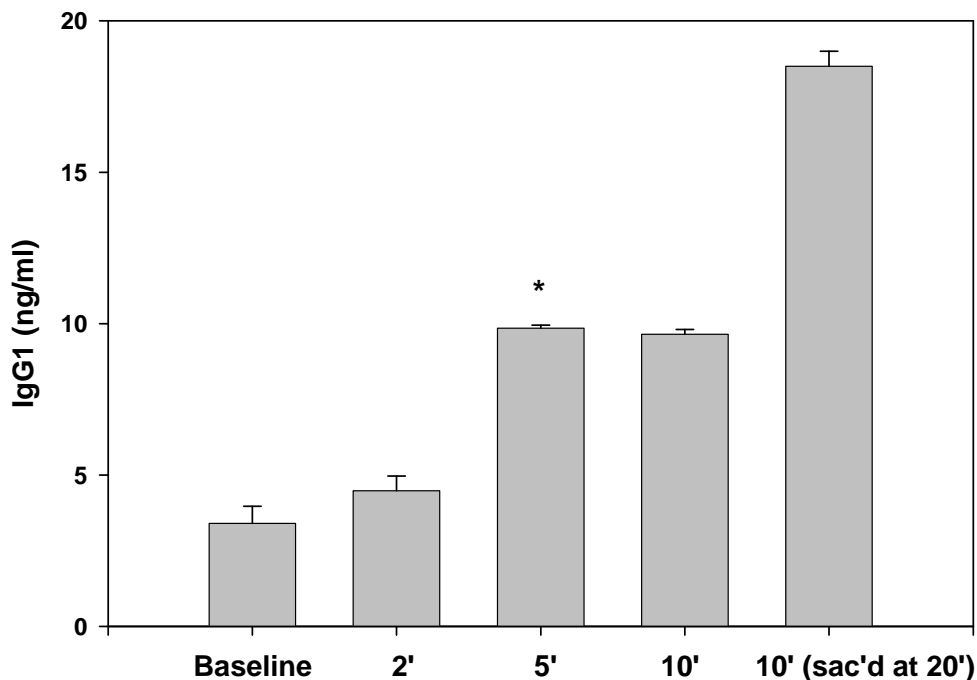
	Baseline	10' mist
Mean:	2.9166	589.5732
SE:	0.8	298.9931
N:	3	3

***significantly higher than baseline, $p < 0.01$**

Uptake of IgG1 in the optic nerve and serum following its application in a formulation containing 1% saponin using the device:

The application of mouse IgG1 to rat eyes for 2, 5, or 10 minutes resulted in its accumulation in the optic nerve (see **Figure 13** below). Levels were highest in rats that were sacrificed 20 minutes after the application of IgG1 for 10 full minutes (bar at far right in figure). It should be noted that the data illustrated by the two bars furthest to the right were obtained from only two rats each.

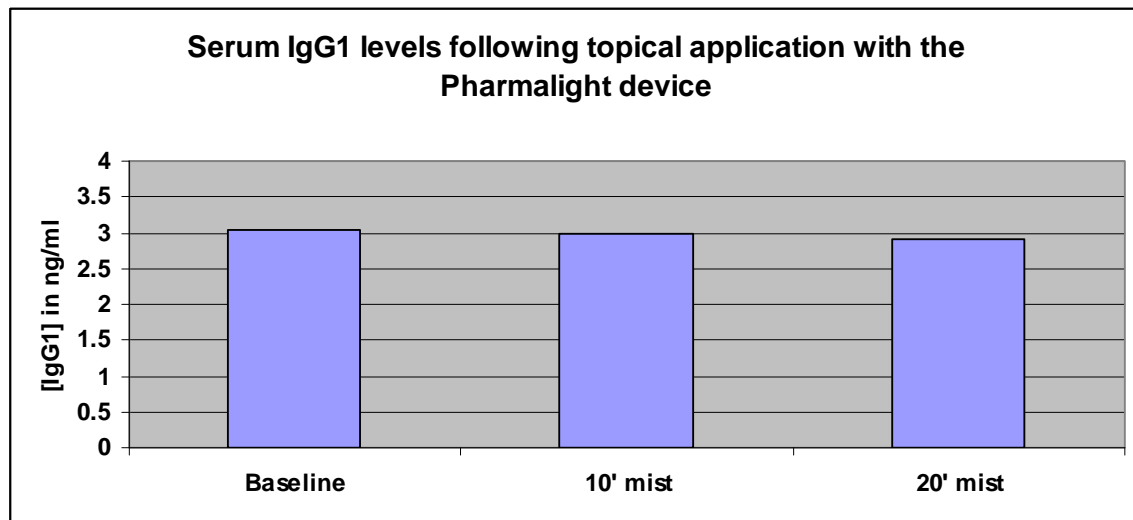
Mouse IgG1 levels in the optic nerve of rats, 10 min after they were subjected to the ocular application of IgG1 for 2, 5, or 10 min with the device - rats were sacrificed at 10 min (except final bar that represents data from rats sacrificed at 20 min)



	Baseline	2'	5'	10'	10'(sac'd 20)
Means:	3.4000	4.4750	9.8500	9.6500	18.500
SE:	0.5612	0.4905	0.0957	0.1500	0.5000
N:	4	4	4	4	2

***significantly higher than baseline, p<0.05**

Serum IgG1 levels were unchanged relative to baseline following application with the PharmaLight device for 10 or 20 minutes (see **Figure 14** below).



Lack of ocular irritation with the device compared to eye drop alone:

Conscious rats that were treated with eye drops containing 1% saponin clearly displayed symptoms of ocular irritation that included a red eye, ptosis, and rubbing. On the other hand, when the same formulation was delivered with the device, the animals did not display signs of irritation.

Discussion

With the dawn of new therapeutic agents for the treatment of posterior segment disease, come new challenges to developing safe and effective methods of drug delivery. Presently, the delivery of pharmaceuticals to the posterior segment of the eye is limited to invasive measures carrying significant risks of serious side effects. The topical administration of pharmaceuticals destined for the posterior segment is faced with many challenges. The drug must first traverse the many ocular compartments/tissues each with its own unique permeability and solubility characteristics. Additionally, topically applied drugs are subject to rapid elimination and systemic absorption (Stjernschantz & Astin, 1993; Patton & Francoeur, 1978; Frishman et al., 2001). The PharmaLight device is designed to deliver topical pharmaceuticals to the ocular posterior segment safely, effectively, and efficiently.

In our study we examined the feasibility of delivering large molecular weight peptides, such as leptin, to ocular tissues including the posterior segment using the PharmaLight device. We also examined whether device-delivered drugs enter systemic circulation in a manner similar to that seen following eye drop application, and whether the device could deliver an irritating formulation in a non-irritating manner.

In the absence of a permeation enhancer (1% saponin), serum leptin levels did not increase significantly in animals following leptin eye drop administration nor following leptin administration with the PharmaLight device. The sera and ocular tissues were measured up to 20 minutes post delivery. However, when saponin was used, serum leptin levels increased significantly following eye drop administration, but did not increase following leptin administration with the device. Additionally, significantly

increased serum leptin levels were found 10 minutes after prolonged exposure of the eye to leptin in the absence of saponin, but was not significantly increased with prolonged exposure using the device. While it has long been accepted that a large percentage of topically applied pharmaceuticals are rapidly eliminated from the ocular surface and systemically absorbed through the vascularized mucosa lining the nasolacrimal drainage system, this was not observed with the PharmaLight technology. This suggests that the PharmaLight device could potentially reduce the risk of potential systemic side effects commonly associated with topical drug delivery systems.

We previously reported that topical eye drop application of low molecular weight peptides not only accumulate in the ocular posterior segment tissues, but also in the CSF (Koevary et al., 2004). In this study, CSF leptin levels were significantly increased following leptin application with the PharmaLight device but not following the application of leptin eye drops; in both of these instances, the formulation contained saponin. This increase in CSF leptin levels was not observed without the use of saponin, suggesting that permeation enhancers may be required in order for large peptides to access the CSF and CNS tissues following device delivery. Importantly, serum levels were not changed relative to baseline, suggesting the increased CSF leptin levels was achieved solely through ocular transport.

Not unexpectedly, 10 minutes after leptin eye drop application to the ocular surface (without the use of saponin), significant levels of leptin was observed in the aqueous humor shortly after leptin eye drop application. Interestingly, however, this increase was not observed 10 minutes after device delivery of leptin. Serum levels of device delivered and eye drop delivered leptin were unchanged relative to baseline.

These findings suggest that leptin delivered by the eye drop versus the device application may be gaining access to the posterior ocular tissues through different transport and absorption routes.

Retinal levels of leptin rose significantly following both eye drop and device treatment compared to baseline. This increase of leptin levels by both delivery methods was seen regardless of whether or not saponin was included in the formulation. In light of the fact that serum leptin levels did not increase in these animals following treatment, it is likely that systemic uptake of leptin was not the source of leptin found in these tissues.

The effects of variable device application times were examined in order to determine whether there was a relationship between application time and tissue uptake. Leptin levels were examined in the retina, sclera and optic nerve following the treatment of rats with leptin with the device for variable amounts of time. Our data showed that leptin levels in the optic nerve were only significantly increased after a two minute device-delivered leptin application. Application times less than two minutes resulted in leptin levels that were too low to be measured in our ELISA assay, while two minute applications resulted in significant leptin accumulation not only in the optic nerve but also in the sclera. Not surprisingly, both optic nerve and scleral leptin levels were notably greater than retinal levels with a two minute device application. While significant leptin levels were achieved in the retina with a two minute application time, this was markedly less than the levels seen in the scleral tissues. This finding is likely due to physiological penetration barriers possessed by the tight junctions of the retinal pigment epithelium (Hughes, Orest, and Joan-En, 2005). Prolonged exposure to device

delivered leptin (10 minute application time) resulted in a similar increase in leptin levels above the two minute application time as seen in scleral and tissues, though the absolute levels were lower.

Scleral and conjunctival tissues possess greater permeability characteristics than the cornea and were found to be less affected by molecular size (Koevary et al., 2004). Some studies have shown that compounds as large as 150 kDa, such as IgG, can penetrate the sclera (Koevary et al., 2004). This information, coupled with the fact that scleral uptake of leptin was fairly significant, led us to determine whether the PharmaLight device could deliver large molecular weight monoclonal antibodies such as IgG. We specifically decided to examine whether the device could deliver mouse IgG1 to the posterior segment since this antibody isotype is identical to that of Avastin and Lucentis. As described in detail in the Introduction, current research has focused on the development of monoclonal antibodies and antibody fragments designed to block the mediators of retinal and choroidal neovascularization, particularly VEGF. Lucentis (ranibizumab), an antibody-based (IgG1) Fab fragment with a molecular weight of 48KDa, was shown to reduce the proliferation of choroidal neovascular membranes (Garner, 1994). In our investigations, we found that IgG1 delivered with the PharmaLight device accumulated in the optic nerve proportional to the application time. Interestingly, serum levels in treated animals were not significantly different from control rats supporting the notion that IgG1 in the optic nerve came from the ocular surface. This preliminary data suggests that significant levels complete and fragmented therapeutic antibodies may be efficaciously delivered to the posterior segment using the PharmaLight device. Of course, more studies in which the retina is investigated will need to be

performed, as well as a more complete pharmacokinetic profile, though our data to date are promising.

Conclusion

With the multitude of challenges facing pharmaceutical drug delivery to the posterior segment, a number of novel approaches to drug delivery have evolved over the years. These studies have been primarily aimed at sustained release intravitreal delivery systems such as nanoparticles, liposomes, and microcapsules (Janoria, Gunda, and Boddu, 2007). Even these techniques are faced with challenges such as short-shelf life, sterilization complications, and invasive measures. Additionally, while these techniques have resulted in substantial vitreal concentrations, the larger particles accumulated only in the vitreous and trabecular meshwork, even after one month post administration (Janoria et al., 2007). With the PharmaLight device we were able to show significant accumulation of large molecular weight peptide and monoclonal antibodies in the posterior segment tissues without increased serum levels. While increased peptide levels were also seen with eye drop administration, serum levels were also increased suggesting a higher risk for systemic side effects. The PharmaLight device, therefore, may offer a non-invasive, effective and safe method for the delivery of pharmaceuticals to the posterior segment for the treatment of ocular disease such as ARMD and diabetic retinopathy.

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