INTRAVITREAL DELIVERY OF CORTICOSTEROID NANOPARTICLES

By

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To my parents and my husband

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Age-related macular degeneration and diabetic retinopathy occurring in the posterior region of the eye are the leading causes of blindness among the elderly. Treatments for these diseases currently include laser photocoagulation and photodynamic therapy. However, aforementioned methods help in eliminating only the existing neovascularization but do not treat the cause of the disease resulting in reoccurrence. Recently corticosteroids are being used to treat posterior ocular diseases due to their angiostatic and antipermeable properties. The rationale for the use of corticosteroids for these conditions is their ability to inhibit growth factors like vascular endothelial growth factor (VEGF). Drug delivery to the posterior segment of the eye is very challenging. One of the major concerns with intravitreal delivery is that repeated injections are disagreeable and lead to further complications. Sustained release systems including microspheres, liposomes and other implant devices offer an excellent alternative to multiple intravitreous injections.

The development of drug delivery systems is becoming important in the treatment of vitreoretinal diseases not only to facilitate drug efficacy but also to attenuate the side effects. These systems can enhance the permeation of the drug, help in controlled release of the drug. Biodegradable poly(lactide-co-glycolide) corticosteroid nanoparticles were prepared and

characterized for size, drug encapsulation and *in vitro* drug release. In order to obtain nanoparticles of desired size and drug loading, it is important to understand the effect of various formulation variables on particle characterization. Here we studied the effect of polymer, drug and surfactant concentration. Increasing the polymer concentration increased the size of the particles. While increase in surfactant concentration decreased the size of the particles and also drug loading.

Cell culture studies were conducted to understand the effect of the corticosteroid nanoparticles on cell toxicity, cell uptake and VEGF secretion. Decrease in toxicity was seen with nanoparticles and use of soft steroid. The uptake of the particles was time and concentration dependent.

Finally, we investigated the efficacy of intravitreal corticosteroid loaded nanoparticles on experimental choroidal neovascularization in a laser-induced mice model. The corticosteroid nanoparticles showed a significant decrease in neovascularization area compared to the control indicating that corticosteroid-loaded polymer nanoparticles can inhibit the development of experimental choroidal neovascularization.

CHAPTER 1
INTRODUCTION

Structure of the Eye

Eye is a complex sensory organ that has a distinct environment. Figure 1-1 shows the structure of the eye. The eye can be divided into two main compartments the anterior segment and the posterior segment [1]. The anterior segment of the eye includes the cornea, iris, ciliary body, lens and consists of two fluid filled spaces: the anterior chamber and the posterior chamber that are filled with aqueous humor which is a thick watery substance being continually produced by the ciliary body and provides the nutrients to the surrounding structures. The posterior segment of the eye consists of three layers, sclera, choroid and retina, surrounding the vitreous cavity, which is filled by the vitreous.

Sclera is a dense, fibrous, viscoelastic connective tissue that forms the outer coat of the eye [2]. The vitreous humor is a transparent gel and is composed of hyaluronic acid and collagen [3, 4] in 98% water. The functions of the vitreous include protecting the eye during mechanical trauma and providing adequate support to the retina [5]. The retina, which is separated from the choroid by Burch's membrane, is the sensory inner coat of the posterior segment of the eye. One side of the retina contains the rod and cones and is adjacent to the retinal pigment epithelium (RPE) and the choroid; the other side of the retina faces the vitreous. Figure1-2 shows the cross section of retina. Photoreceptor cells are located in the subretinal region near to the choroid. The macula is the center part of retina.

Blood–retinal barrier (BRB) composed of the retinal vasculature and the retinal pigment epithelial cells separates the retina and the vitreous from the systemic circulation and vitreous body. Two types of transmembrane proteins, occludin and the family of claudins, are responsible for the direct cell-to-cell attachment in the establishment of the tight junction barrier [6, 7]. The

choroid is vascular and pigmented tissue between the retina and the sclera. The retinal pigment epithelium (RPE) is located between the choroid and the retina.

Ocular Diseases

Millions of people suffer from various sight threatening ocular conditions such as diabetic retinopathy, age related macular degeneration (AMD), cataract and glaucoma. Table 1-1 shows the statistics on number of people suffering from various ocular conditions in US. Among these diabetic retinopathy and age related macular degeneration occurring in the posterior segment of the eye are the leading causes of blindness in developed countries.

Diabetic retinopathy is a complication of diabetes mellitus. It is characterized by the breakdown of blood-retinal barrier. Hyperglycemia changes the formation of the blood-retinal barrier and also make retinal blood vessel become more permeable [1]. As the disease progresses neovascularization occurs due to lack of oxygen, forming fragile blood vessels. These newly formed blood vessels cloud vision and bleed and damage the retina, resulting in increased vascular permeability leading to edema [8] and finally resulting in vascular proliferation [9].

Age-related macular degeneration (AMD) is a disease associated with aging that gradually destroys sharp, central vision. AMD affects the macula, the part of the eye that allows us to see fine detail. Choroidal neovascularization is associated with macular degeneration. Choroidal (or subretinal) neovascularization (CNV) characteristic of macular degeneration is a major cause of visual loss. Choroidal neovascularization originates from choroidal vessels. It is accompanied by fluid and blood breaking through the Bruch's membrane into the subretinal pigment epithelial space and/or into the subretinal space, resulting in irregular elevations of the surface of the retina.

These diseases are mainly treated with laser photocoagulation. Laser photocoagulation destroys neovascularization in age related macular degeneration and decreases vascular leakage

and destroys new blood vessels in diabetic retinopathy. In this procedure leaking blood vessels are burnt by the laser. Laser photocoagulation does not help in restoring the lost vision and has to be repeated several times in many patients. It can also lead to permanent damage of the retina and not effective for long term treatment. Therefore, new therapeutic approaches are being sought for these conditions. Table 1-2 shows the various therapeutic agents for retinal diseases.

Recent preclinical studies have suggested that pharmacologic intervention or anti-angiogenesis therapy may be useful to treat various forms of ocular neovascularization. Much of this work has focused on blocking vascular endothelial growth factor (VEGF) [10-13] which has been shown to play a major role in initiating these diseases.

Role of Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen and appears to be important in the development of ocular neovascularization. Retinal cells forming the outer layer of BRB express VEGF [14]. It has been implicated in the onset and progress of diabetic retinopathy and choroidal neovascularization secondary to age related macular degeneration [15]. Studies in humans showed increased VEGF in aqueous humor and vitreous of patients with diabetic retinopathy [16-19]. Animal studies have shown that injection of VEGF into the vitreous can cause a diabetic retinopathy like state [20].

Recent evidence also suggests a central role of VEGF in the development of choroidal neovascularization. Excised human choroidal neovascularization after experimental submacular surgery have shown elevated VEGF levels [21]. Vitreous VEGF levels were found to be significantly higher in patients with age-related macular degeneration and choroidal neovascularization as compared to healthy controls [19]. VEGF also increases phosphorylation of tight junction proteins, such as occludin resulting in the breakdown of the blood retinal barrier

[22, 23]. VEGF also plays a major role in inflammation by inducing intercellular adhesion molecule (ICAM-1) expression and leukocyte adhesion.

The above information shows VEGF to be a good target for pharmaceutical intervention. Recent studies showed that anti-VEGF therapy may inhibit breakdown of blood retinal barrier induced by diabetes in animals [24].

Several researchers showed that macromolecules such as VEGF antibodies and aptamers to inhibit retinal vascular changes [25, 26]. Studies have shown regression or prevention of neovascularization in retina [25, 27, 28] and choroid [26] in several animal models (primate [29], mouse [28] and rat [20]) using anti-VEGF aptamers [25] and antibody fragments agent [26]. But very little research with regard to low molecular weight compounds has been done. Corticosteroids are low molecular weight lipohilic compounds that are easier to administer and stable. They offer an alternative to the macromolecules due to their anti angiogenic and antipermeable properties.

Corticosteroids in Ocular Delivery

Corticosteroids are traditionally used due to their anti inflammatory property, but recently they gained interest for treating posterior ocular conditions due to their angiostatic and antipermeable properties [30]. Corticosteroids show their effect by binding to the steroid receptors present in the cells. They then act by either induction or repression of the target genes and inhibit inflammatory symptoms like edema and vascular permeability [31].

Posterior ocular diseases are characterized by edema and neovascularization. Corticosteroids show their effect by action on various targets. Edema is mainly caused due to the breakdown of blood-retinal barrier and VEGF plays a critical role in causing the leakage. Corticosteroids act on VEGF directly by inhibiting VEGF secretion [32, 33] and also by countering the effects of VEGF, inhibiting cytokine production, attenuating leukocyte adhesion.

and inducing apoptosis [34]. Corticosteroids also affect the distribution of the tight junction proteins increasing their expression and also can reverse the phosphorylation of occludin (a tight junction protein) caused by VEGF [35].

Corticosteroids are also potent inhibitors of neovascularization. Corticosteroids act by inhibiting the basic fibroblast growth factor induced migration and tube formation [36], they further act by inhibiting extracellular matrix turn over by down regulation of metalloproteinase-2 production and down regulation of ICAM-1 expression [37] they also act by decreasing VEGF levels [32, 38] and decreasing the expression of major histocompatability complex-11 expression [39].

Routes of Drug Administration

Traditional routes of administration of steroids are topical, systemic and periocular (including subconjunctival, sub-tenon's and retrobulbar), refer to Figure 1-3. The topical route using eye drops constitute approximately 97% of total formulations into the eye, but therapeutic concentrations are not achieved by this route mainly due to lacrimation and presence of barriers. Although drug delivery to the posterior segment can be achieved by the systemic route, large doses are required to achieve therapeutic concentration due to the presence of blood ocular barriers which can result in side effects. Periocular injections minimize most systemic side effects and eliminate the need for daily patient compliance, yet the blood ocular barrier can still impede from this route from attaining therapeutic concentrations to its intended target tissue. Due to the poor accessibility, effective treatment of diseases related to the posterior segment of the eye, the development of new delivery systems, routes (intravitreal) and new corticosteroids will help to achieve high angiostatic and antipermeability concentrations and to reduce the adverse effects [30].

Intravitreal injection provides the most direct approach in delivering drugs to the posterior segment, and therapeutic tissue drug levels can be achieved. Intravitreal delivery allows for sufficiently high local concentrations of corticosteroids to maximize their anti-inflammatory and angiostatic effects and attenuating adverse effects. Intravitreal injection of triamcinolone acetonide (Kenalog-40) is routinely used in practice to treat diseases in the posterior region of the eye [40, 41]. The success of intravitreal triamcinolone acetonide therapy has led to its use in a variety of diseases such as exudative macular degeneration and proliferative diabetic retinopathy (PDR) due to their potent antiangiogenic action.

One of the most common adverse effects seen with corticosteroids is ocular hypertension [39, 41-43]. In one study, intravitreal injections of 25 mg of triamcinolone acetonide resulted in ocular hypertension in approximately 50% of eyes, commencing 1 to 2 months after the injection. Several large studies using 4 mg of triamcinolone showed an elevated pressure incidence in 30% of patience. The factors for developing ocular hypertension are not yet understood [42]. Recent studies showed that corticosteroid treatment of human trabecular meshwork cells produced delayed, progressive cellular and extracellular glycoprotein induction [31]. Cataract is also a commonly seen complication caused by chronic use of corticosteroid treatments. Several mechanisms such as: formation of covalent adducts with the steroid molecules with lysine residues of lens, lowered ascorbic acid in the aqueous humor and metabolic changes like altered phospholipids metabolism were shown to cause cataract. Because of their potential side effects a novel approach to the use of corticosteroids is highly desirable. This can be achieved by either developing soft drugs or by application of slow release systems. The development of soft drugs helps to reduce the risk of undesired side effects. The use of soft drugs is an effective way to prevent side effects as they metabolize at the site of action or at the

site of application. Loteprednol etabonate is a site-active corticosteroid synthesized through structural modifications of prednisolone related compounds so that it will undergo a predictable transformation to an inactive metabolite [44]. Loteprednol etabonate was effective in the treatment of giant papillary conjunctivitis, seasonal allergic conjunctivitis, postoperative inflammation and uveitis. In a large double-blind study on corticosteroid responders loteprednol etabonate demonstrated less propensity to cause clinically significant elevation in intraocular pressure when compared to prednisolone acetate [45].

Drug Delivery Systems

Posterior ocular diseases are chronic in nature and require multiple injections to maintain therapeutic concentrations, which are not only disagreeable but also increase the risk of cataract formation and retinal detachment with the frequency of injection. Sustained drug delivery devices are an excellent way to avoid multiple intravitreal injection. The use of drug delivery systems (DDS) is an effective way of delivering drugs to the posterior region of the eye for an extended period. Table 1-3 shows the various drug delivery systems either clinically approved or under research for posterior ocular conditions. DDS can improve the permeation, help in prolonged release of the drug and also helps to attenuate the adverse effects. Recently implants of ganciclovir are being used, but surgery is required for placing the implant and its removal. Polymeric nanoparticles and microparticles offer an excellent way of sustained drug delivery [46, 47]. As smaller particles are better tolerated in the eye, we chose nanoparticles for drug delivery [46, 48]. Particles 200 nm or less are shown to be localized in the RPE cells [46]. Nanoparticle formulation is one of the strategies currently used to improve drug absorption across biological membranes [49]. In order to achieve sustained drug release and prolonged therapeutic effect using ophthalmic drug-loaded nanoparticles, the entrapped drug must be released from the nanoparticles at an appropriate rate. If the release rate of the nanoparticle

formulated drug is too fast it may fail to provide sustained exposure while too slow a release rate could prevent the drug from reaching a sufficient concentration. In drug-loaded nanoparticles, the active molecules are confined within polymeric matrices by relatively strong noncovalent interactions such as ionic, hydrogen-bonding, hydrophobic, or dipole [50-52]. Biodegradable polymers such as poly(lactic-co-glycolic acid) and poly(lactic acid) are often used for the preparation of nanoparticles. They are aliphatic polyesters derived from glycolic acid and from lactic acid enantiomers. These polymers are biocompatible and can be synthesized in various molecular sizes allowing the encapsulation of specifically adapted formulations. They degrade by hydrolysis of the ester linkages of polymer backbone and form lactic acid and glycolic acid and are eliminated from the body by Krebs's cycle through normal excretion.

Poly(lactide-co-glycolide) is widely used for surgical dressings fracture repairs and dental repairs. Several researchers have used poly(lactide-co-glycolide) for the preparation of DDS such as implants and nano/microspheres for controlled drug delivery. The degradation rate of these polymers can range from months to years depending on the molecular weight, conformation and copolymer composition [53].

Hypothesis

We hypothesize that sustained-release corticosteroid nanoparticles can improve the therapeutic profiles of corticosteroids for posterior ocular diseases. Our hypothesis will be tested by the following aims:

- Prepare and characterize corticosteroid nanoparticles for: size, shape, drug encapsulation and *in vitro* release.
- Investigate the effect of steroids and their nanoparticles on VEGF expression, cellular uptake and cytotoxicity using ARPE-19 cells.
- Conduct *in vivo* study in mice and study the effect of corticosteroid nanoparticles on laser induced choroidal neovascularization in mice.

Table 1-1. Eye disease prevalence and projections in adults 40 years and older in the U.S

Eye Disease	Current Estimates (in millions)	2020 Projections (in millions)
Advanced age-related macular degeneration (with associated vision loss)	1.8*	2.9
Glaucoma	2.2	3.3
Diabetic Retinopathy	4.1	7.2
Cataract	20.5	30.1

Table 1-2. Treatments being considered for AMD and DR

Drug Class	Drug
Photodynamic therapy	Visudyne [54]
VEGF Inhibitors Anti-VEGF aptamer Anti- VEGF antibodies Anti-VEGF antibody fragments VEGF-trap	 Pegaptanib [25] Bevacizumab [55] Ranizumab [55] VEGF-trap [56]
PKC-ß inhibitor	Ruboxistaurin [55]
Corticosteroids	Flucinolone acetonide [57] Dexamethasone [58] Triamcinolone acetonide [59] Anacortave acetate [60]

Table 1-3. Drug delivery systems for treating posterior ocular conditions

Drug delivery system	Drug
Implants	Ganciclovir [61-63] Flucinolonee acetonide [57, 64] Dexamethasone [65] Betamethasone phosphate [66, 67] Fluconazole [68] Triamcinolone [69]
Cyclodextrins	Dexamethasone [70, 71]
Liposomes	Cidofovir [72]
Ionthophoresis	Methotrexate [73] Dexamethasone [74]
Nano/microparticles	Budesonide [48] Cyclosporine [75] Peroxicam [76] PKC412 [56]
Transdermal	Prednisolone [77]

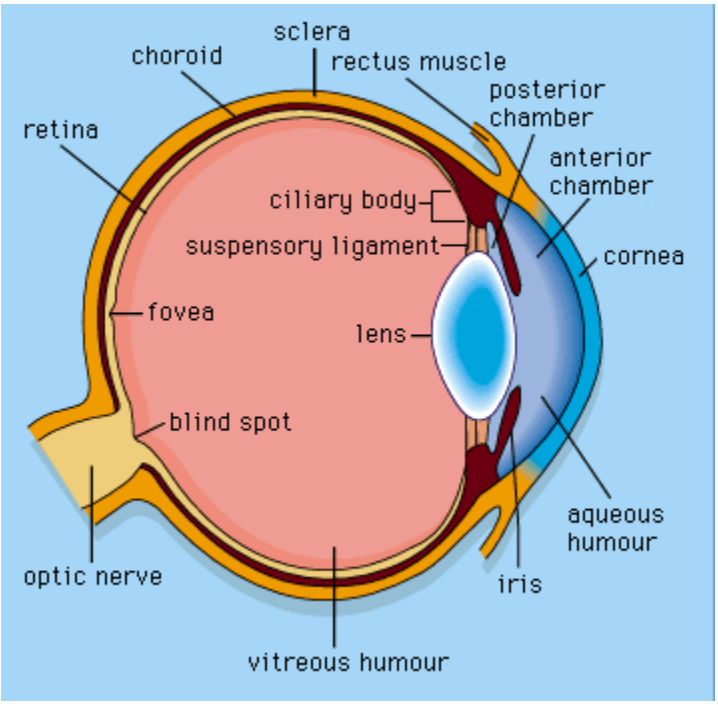


Figure 1-1. Structure of the Eye

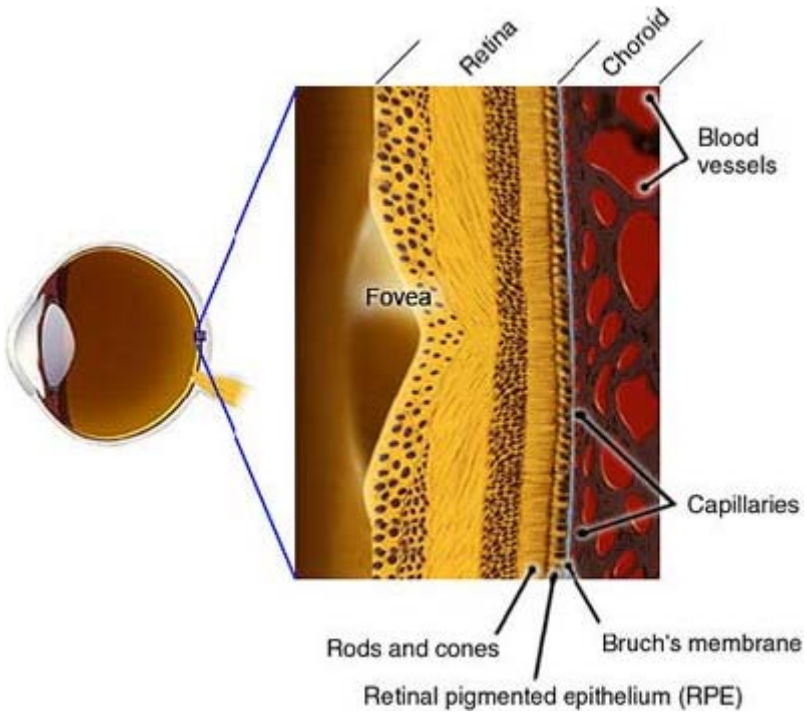


Figure 1-2. Detailed structure of the retina

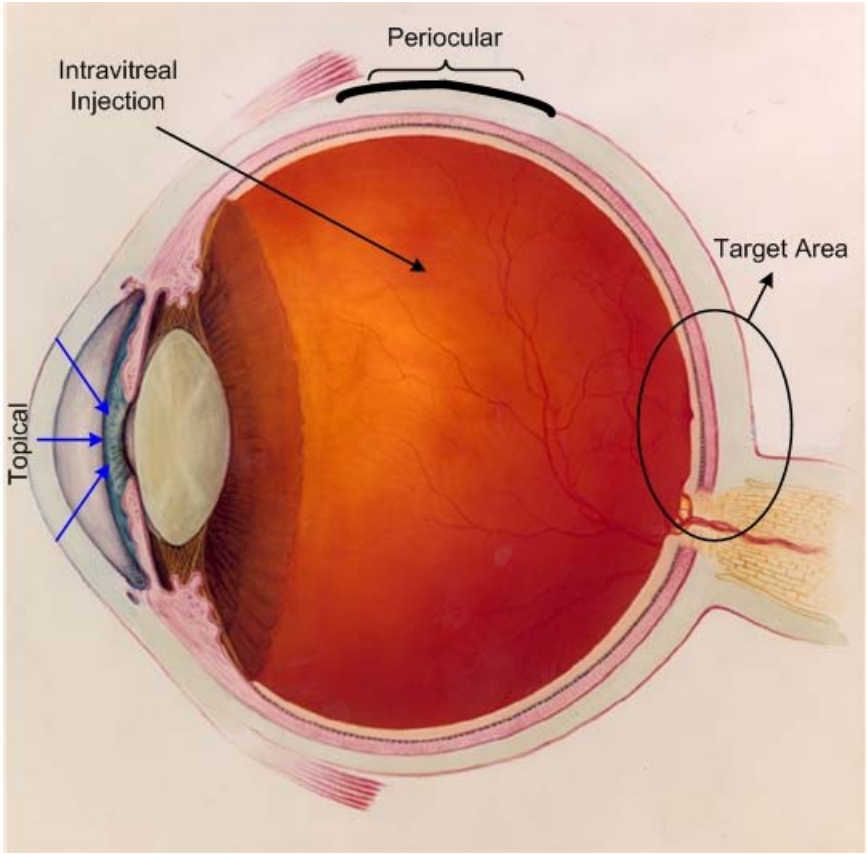


Figure 1-3. Routes of drug delivery to target posterior region of eye

CHAPTER 2
PREPARATION AND CHARACTERIZATION OF TRIAMCINOLONE ACETONIDE NANOPARTICLES

Introduction

Drug delivery to the posterior region of the eye remains a challenge. Drug delivery systems are becoming popular recently as a suitable way to sustain drug release. They have the advantage of delivering therapeutic agent to its site of action at an optimal rate. Slow release delivery systems can alter the drugs biopharmaceutics [78]. They also can decrease toxic side effects due to slow drug release and also eliminate the inconvenience of repeated administration. Various drug delivery systems include: liposomes, implants, microparticles and nanoparticles. Several researchers showed the effectiveness of these systems for ocular drug delivery [79].

Recently there has been interest in the use of nanoparticle drug delivery to the posterior region of the eye. Nanoparticles are among the most widely studied colloidal systems over the past two decades. Nanoparticles are polymeric particles, ranging from 10 nm to 1 μm, in which the drug is dissolved, entrapped, encapsulated or adsorbed [49]. They have the advantage of easy administration by injection due to their small size. Unlike polymer implants, nanoparticles do not require surgical procedures for implantation and removal. Nanoparticles have been shown to be efficient as ocular drug delivery systems by prolonging the duration of the action of drugs. Bourges et al. examined intravitreal administered nanoparticles in rat eyes [46]. Poly(lactic-co-glycolide) nanoparticles injected into the vitreous cavity of rats, were found within the vitreous cavity immediately after intravitreal injection. By 6 hours, the majority of the nanoparticles were found within the retinal pigment epithelium (RPE) and by 24 hours, a significant concentration of nanoparticles was observed in the cytoplasm of the RPE. With the rat model, intra cytoplasmic retinal cell concentrations of nanoparticles remained elevated as far as 4 months after a single intravitreal injection [46].

Nanoparticles are made of natural or artificial polymers that are biocompatible. The various polymers used to fabricate nanoparticles are polyacrylamide, polymethylmethacrylate, poly(lactide-co-glycolide) and E-caprolactone [79]. Biodegradable nanoparticles formulated from poly(lactide-co-glycolide) and poly(vinyl alcohol) (PVA) polymers have been extensively investigated for various drug delivery applications. Poly(lactide-co-glycolide) is biocompatible, and more importantly, the degradation rates of polymer and the accompanying release of encapsulated drugs can be controlled by the polymer's physicochemical properties, such as molecular weight, hydrophilicity, and the ratio of lactide to glycolide [80].

Rate of drug release from controlled release formulations depends on the characteristics of the particles, including particle size, size distribution, drug content, incorporation and surface morphology [81]. The particle size and drug content are particularly important characteristics that determine drug release. These characteristics depend on the specific formulation parameters [82]. To control these, it is important to study the effect of processing and materials parameter on particle size and drug content of the nanoparticles. Our goal was to prepare corticosteroid nanoparticles with biodegradable polymer poly(lactide-co-glycolide) that can sustain drug release.

In this study, we developed nanoparticles by an emulsion solvent evaporation technique using sonication. Initially an O/W emulsion was formed with the aqueous phase containing the emulsifier and organic phase in which the drug and the polymer are dissolved. The organic phase is then evaporated from the emulsion droplets resulting in the formation of nanoparticles which are collected through centrifugation. The formulation parameters: amount of poly(lactide-co-glycolide) polymer, triamcinolone acetonide drug and poly(vinyl alcohol) surfactant were varied to study the effect on size, encapsulation and drug release.

Materials and Methods

Materials: Poly(lactide-co-glycolide) (50:50) inherent viscosity of 0.68 dL/g was obtained from Lactel absorbable polymers (Pelham, AL), polyvinyl alcohol (PVA) was obtained from Sigma Chemical Co. (St. Louis, MO), HPLC grade methylene chloride/dichloromethane , acetonitrile were obtained from Fisher Scientific, dialysis membrane bags for *in vitro* release studies (molecular weight cut off 10,000; spectrum Laboratory (Rancho Dominguez, CA).

Nanoparticle Preparation

Nanoparticles were prepared by using solvent evaporation method [83]. The drug and the polymer were dissolved in organic phase, and this solution was added to 10ml of 1% aqueous poly(vinyl alcohol) (PVA) solution. The resultant mixture was sonicated at 40W for 1 minute with a probe sonicator (Sonics vibracell, Newtown, CT), to obtain an O/W emulsion. The O/W emulsion was then added to remaining 40ml of 1% aqueous PVA solution in a 50 ml conical flask. The contents were stirred at 250 rpm on a magnetic stirrer at room temperature overnight to evaporate the organic phase, allowing the formation of turbid particulate suspension. The nanoparticles were separated by ultracentrifugation (Beckman Coulter, Inc.Fullerton, CA) at 35,000g for 1hour. The pellets were washed three times in double distilled water and freeze dried with Freezone 6 lyophilizer (Labconco Corporation, Kansas City, MO) to obtain lyophilized particles. [84]. Figure 2-1 shows the nanoparticle preparation process.

Nanoparticle Characterization

The particle size of the corticosteroid nanoparticles was determined with a NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA). Sample of polymeric nanoparticles 5 mg were suspended in 5 ml of double distilled water, and the diluted suspension was subjected to particle size measurement. The morphology of the nanoparticles was analyzed by scanning electron microscope (SEM) JEOL JSM-6335F instrument (Major Analytical Instrument Center

(MAIC), UF, Gainesville, FL). A small amount of the freeze dried sample was layered on the SEM stubs and coated with carbon in a high-vacuum evaporator. The coated samples were then observed for their surface morphology with the instrument set at 15 kV.

Turbidity test was performed between the micronized triamcinolone acetonide and its nanoparticles by suspending them.

Drug Encapsulation Efficiency

Encapsulation efficiency of the particles was determined by weighing 5 mg of the freeze dried particles in a glass tube. Methylene chloride 2 ml was added and mixed thoroughly at room temperature. The resultant solution was evaporated to dryness under vacuum and the dried residue was reconstituted with acetonitrile water mixture (70:30). The reconstituted solution was vortexed for 1minute and centrifuged at 4000 rpm for 15minutes, and 100µl of the supernatant is injected into the HPLC column. The HPLC was performed isocratically at ambient temperature, at a flow rate of 1 ml/min. The mobile phase comprised of acetonitrile and water (70:30 v/v). A Waters C18 column (4.6x150 mm) preceded by a guard column, and detection was accomplished using UV detection at a wavelength of 254 nm. The calibration curve was obtained with standards from 1 to 50 µg/ml and was linear ($r^2 > 0.99$).

The % encapsulation efficiency is calculated using the following equation:

$$\%\text{Encapsulation efficiency} = \frac{\text{Actual amount of drug}}{\text{Theoretical amount of drug}} \times 100$$

$$\%\ \text{Loading efficiency} = \frac{\text{Amount of drug in nanoparticles}}{\text{Total amount of nanoparticles}} \times 100$$

***In Vitro* Release**

In order to avoid the drawback of aggregation during freeze drying the release studies were performed with nanoparticles suspensions immediately after their preparation. The concentration

of the released drug was studied as a function of time. The results over 100 hours are shown. Suspension of corticosteroid nanoparticles containing 300 μg of the drug was transferred into floatable dialysis membrane unit (10,000 mol.wt), and the unit was placed in 50 ml centrifuge tube containing phosphate buffer solution (PBS, pH 7.4) at 37°C. Samples of 1 ml were collected at regular intervals and replaced by PBS. The corticosteroid released was then analyzed using HPLC. Phosphate buffer solution containing the drug was directly injected into the HPLC column and acetonitrile: ammonium acetate buffer (50:50) was used as the mobile phase at a flow rate of 1 ml/min. Calibration curve was obtained with standards from 0.5 μg/ml to 20 μg/ml and was linear (($r^2 > 0.985$).

For release studies between micronized triamcinolone, small nanoparticles of 145 nm and larger nanoparticles of 415 nm, *in vitro* release studies were conducted under sink conditions using 0.5% sodium dodecyl sulfate containing 650 μg. of drug. Calibration curve was obtained with standards from 1 to 20 μg/ml and was linear ($r^2 > 0.99$).

Statistical Analysis

One way ANOVA was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) with p value less than 0.05 considered as significant.

Results

Morphology

Figure 2-2 and Figure 2-3 show the SEM pictures of spherical nanoparticles with smooth surface. Figure 2-4 shows turbidity of nanoparticles compared to micronized triamcinolone acetonide.

Effect of Polymer Poly(lactide-co-glycolide) on Size and Encapsulation

Effect of polymer poly(lactide-co-glycolide) on nanoparticles characteristics is shown in Table 2-1. The polymer content was increased from 50 to 200 mg while keeping all the other processing conditions similar. Increasing the polymer concentration lead to gradual increase in the particle size from 115 to 200 nm. When the polymer amount was increased to 400 mg while keeping the ratio of drug to polymer 1:10, 67% of the particles showed mean concentration of 445 nm while 33% were 150 nm. Figure 2-5 shows the distribution of the particles. Increasing the polymer concentration increases the viscosity of the organic phase and resisting droplet breakdown during sonication. Also, increase in drug encapsulation was seen with increase in polymer concentration in organic phase. The drug encapsulation efficiency increased from 48% to 65%. Increase in polymer increases particle size resulting in more drug being encapsulated.

Effect of Triamcinolone Acetonide on Size and Encapsulation

The effect of drug concentration was studied by increasing triamcinolone acetonide from 5 to 15 mg while maintaining the polymer concentration constant. Table 2-2 shows the observed results. The diameter of the particles remained independent of the amount of the drug present. As the drug concentration was increased from 5 to 15 mg there was a significant increase in the drug loading and encapsulation efficiency. Lower encapsulation was seen with 5 mg of drug. The lower encapsulation might be due to diffusion of the drug from the O/W emulsion globule into the aqueous continuous phase resulting in lower encapsulation.

Effect of Poly(vinyl alcohol) on Size and Encapsulation

Emulsifier plays a critical role in the preparation of nanoparticles by emulsion solvent evaporation method. The amount of surfactant is crucial as it prevents the coalescence of the droplets and protects them. In order to study the effect of surfactant poly(vinyl alcohol) on the nanoparticle properties, the concentration was varied from 1 to 4% while keeping the other

variables standard. The observed results are shown in Table 2-3. As the concentration increased from 1 to 4% there was a decrease in the size of the particles from 172 to 120 nm. Concentration of 4% emulsifier leads to smaller particles due stabilizing function of the surfactant, thus preventing the aggregation of particles. Increasing the PVA concentration decreased the drug encapsulation. This is mainly due to the decrease in particle size caused by increased stability. Decrease in particle size results in larger surface area which leads to increased diffusion of the drug from the O/W emulsion into the continuous aqueous phase. Increase in encapsulation efficiency was observed when the aqueous phase was saturated with the drug, preventing the diffusion of the drug from O/W emulsion into the aqueous phase.

In Vitro Release Studies

Release of corticosteroids from poly(lactide-co-glycolide) nanoparticles into phosphate-buffered saline (PBS) was measured *in vitro* at 37°C. Figure 2-6 to figure 2-9 show the cumulative release data of various formulations. All the formulations showed initial burst release of more than 10% on the first day which might be due to the presence of the drug on the surface of nanoparticles. A drug release of 50% was seen in 24, 34 and 96 hours for micronized triamcinolone acetonide, 145 and 415 nm nanoparticles respectively. The size of the particles plays a crucial role on the drug release. Larger particles showed slower release compared to smaller particles with similar formulation parameters. Since smaller particles have larger surface area, the drug release is faster. At the end of 192 hrs the cumulative release for triamcinolone acetonide micronized, triamcinolone nanoparticles of 145 and 415 nm was 100, 92 and 72% respectively. Figure 2-8 shows the cumulative triamcinolone acetonide released from micronized triamcinolone acetonide and its nanoparticles of different size.

Unlike diffusion through the membrane of a reservoir system, where the rate of drug release is linear with time, matrix controlled diffusion is linear with the square root of time for

spherical structures, such as microspheres and nanospheres. In accordance with this our *in vitro* release data with all the formulations showed better fit with Higuchi equation compared to zero order. Tables 2-4 to 2-7 show the R^2 values of the various formulations calculated with zero order and Higuchi equations.

Figure 2-9 shows the extrapolated *in vitro* data of micronized triamcinolone acetonide, nanoparticles of around 145 and 415 nm particles respectively showing 100% drug release in 5, 7 and 16 days respectively. Figure 2-10 shows the SEM images taken during *in vitro* release studies over a 15 day period. The images show initial swelling of the particles followed by pore formation and adhering.

Discussion

Drug delivery systems help in controlled delivery of therapeutic agents to its site of action and at optimal rate. An ideal controlled-release formulation should release the entrapped drugs in a continuous manner over a desired time period. The results from the different studies show the potential of colloidal systems as ocular drug delivery systems for either hydrophobic or hydrophilic drugs [79].

Poly(lactide-co-glycolide) polymers are natural biodegradable and biocompatible polymers that are most widely used. It is also approved for human use by the Food and Drug Administration. The nanoparticles formulation with a therapeutic agent entrapped into the polymer matrix provides sustained drug release [49]. The lactide/glycolide polymers are cleaved by hydrolysis to form lactic acid and glycolic acid that are eliminated from the body through the Krebs's cycle as carbon dioxide and water [85].

Typical emulsion solvent evaporation process was employed to produce polymeric nanoparticles and good reproducibility with size and encapsulation was observed. The nanoparticles were formed as a result of evaporation of the organic solvent from the emulsion

nanodroplets. The particle size and encapsulation efficiency are important parameters and can affect the biopharmaceutical properties of the nanoparticles. The size can also play an important role in endocytosis of nanoparticles. The smaller the size of the particle, the better is the cellular uptake of particles [86].

The size of the particles was mainly affected by polymer concentration in the organic phase. Our results corroborate with those shown by other researchers that increase in resistance caused by larger amounts of polymer during the formation of emulsion results in larger nanoparticles [81]. The particles prepared were unimodal below 200 mg of poly(lactide-co-glycolide) when the polymer amount was increased to 400 mg, 67% of the particles were around 415 nm while 33% of them were 150 nm (figure 2-5). This formulation needs to be further optimized to prepare larger particles with uniform size to prolong the drug release. This shows that the polymer concentration along with the sonication time and power need to be adjusted to obtain uniform particles of larger size.

Previous studies have shown that factors that prevent the diffusion of the drug into the aqueous phase increase the encapsulation efficiency [87]. In our study, we observed that increase in encapsulation efficiency can be obtained by either increasing the drug concentration or by saturating the aqueous phase with the drug.

Particle size and loading are important characteristics that can affect the release of the drug from the nanoparticles. *In vitro* release studies showed initial burst followed by sustained drug release. Our *in vitro* release profiles are similar to those obtained by Feng et al. [88] who observed a 30 to 40% taxol release at the end of 2 weeks from nanospheres. Based on our drug release profiles and Higuchi plots, it appears that the drug is entrapped in the polymer matrix.

Kompella et al. showed cumulative release of 35 to 50% cumulative budesonide release at the end of 2 weeks from nanoparticles following an initial burst of 15 to 20% [89].

Our results showed a decrease in drug release with larger particles compared to the smaller particles. Smaller particles possess a larger surface area, which in turn can lead to a faster release of the drug incorporated. Yoncheva et al. showed that the release properties of the nanoparticles and their size are interrelated [90]. Smaller nanoparticles also lead to a shorter average diffusion path of the matrix entrapped drug molecules.

The release of the entrapped drug from the polymer matrix has been found to occur through diffusion and degradation mediated process. The release of the drug in the early stage is believed to occur mainly through diffusion in the polymer matrix while in the later phases the release occurs due to both diffusion and polymer degradation [91]. The change in surface morphology of nanoparticles with time following incubation in PBS is shown in figure 2-10. The particles showed roughness and pore formation followed by aggregation of the particles. Similar results were shown by Panyam et al. they also showed that the glass transition temperature (T_g) decreases with the decrease in polymer molecular weight resulting in more softer polymer prone to aggregation resulting in fusion of the particles [92]. Vishwanath et al. [93] showed that PLA and PLGA undergo deformation and aggregation owing to lowering of the polymer glass transition temperature below 37°C following its hydration in the buffer.

The goal of this study was to understand the effect of various formulation parameters on nanoparticle properties and select a formulation suitable for intravitreal delivery *in vivo.* Based on our results, the formulation with drug to polymer ratio of 1:10 with 1% PVA was selected as it gave us unimodal size particles with reproducibility, good encapsulation efficiency and sustained drug release compared with micronized triamcinolone acetonide. It also showed less

turbidity compared to micronized triamcinolone acetonide and as a result may provide better visibility after intravitreal delivery. As the volume that can be injected into the vitreous is limited, higher polymer concentrations were not considered as the loading efficiency decreases with increase in the amount of polymer.

Conclusion

Solvent evaporation method using sonication was successfully used in the preparation of triamcinolone acetonide nanoparticles. Particles produced were in the nanoparticle size range and unimodal, with good drug encapsulation. The various formulation factors affecting the size, loading and release rate were studied. Table 2-8 shows the effect of various formulation parameters on particle characterization. *In vitro* release studies indicate sustained release of the drug following an initial burst. Nanoparticles can sustain drug release for a prolonged period preventing repeated intravitreal injections and hence can be used to treat posterior ocular conditions.

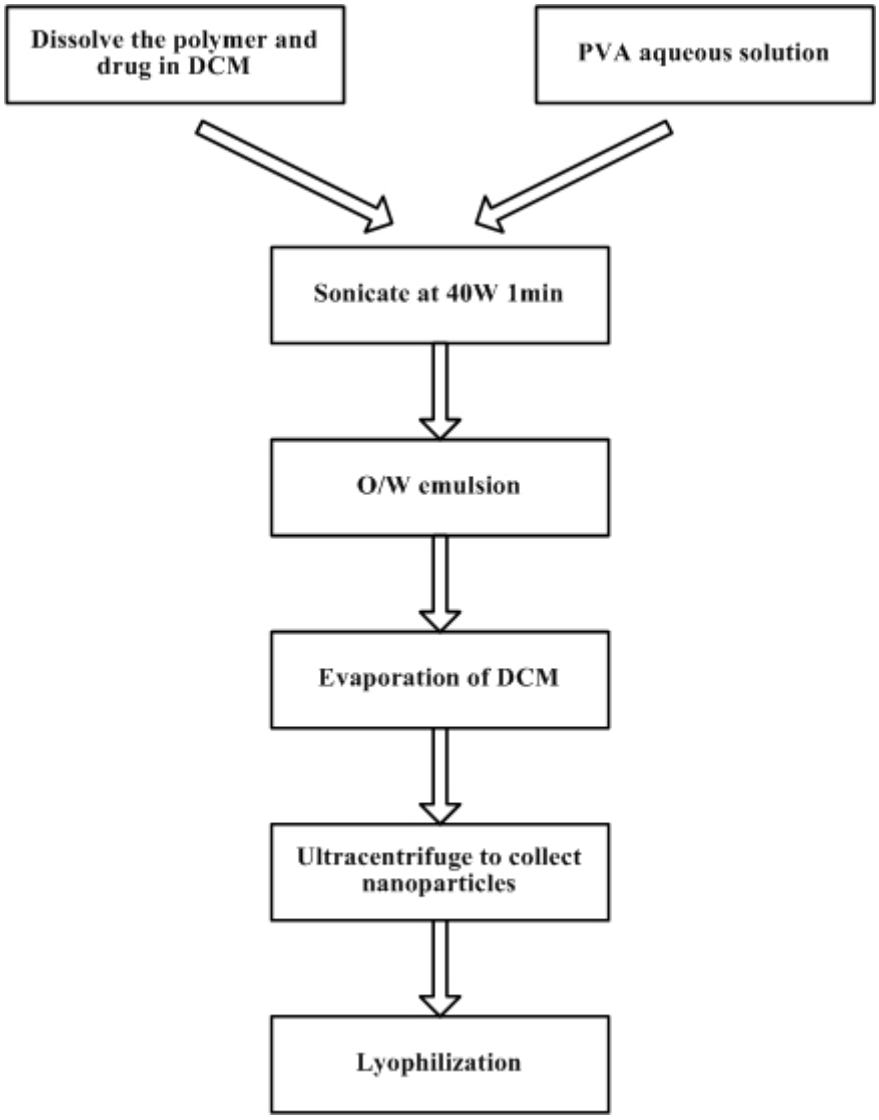


Figure 2-1. Flow chart showing the nanoparticle preparation process

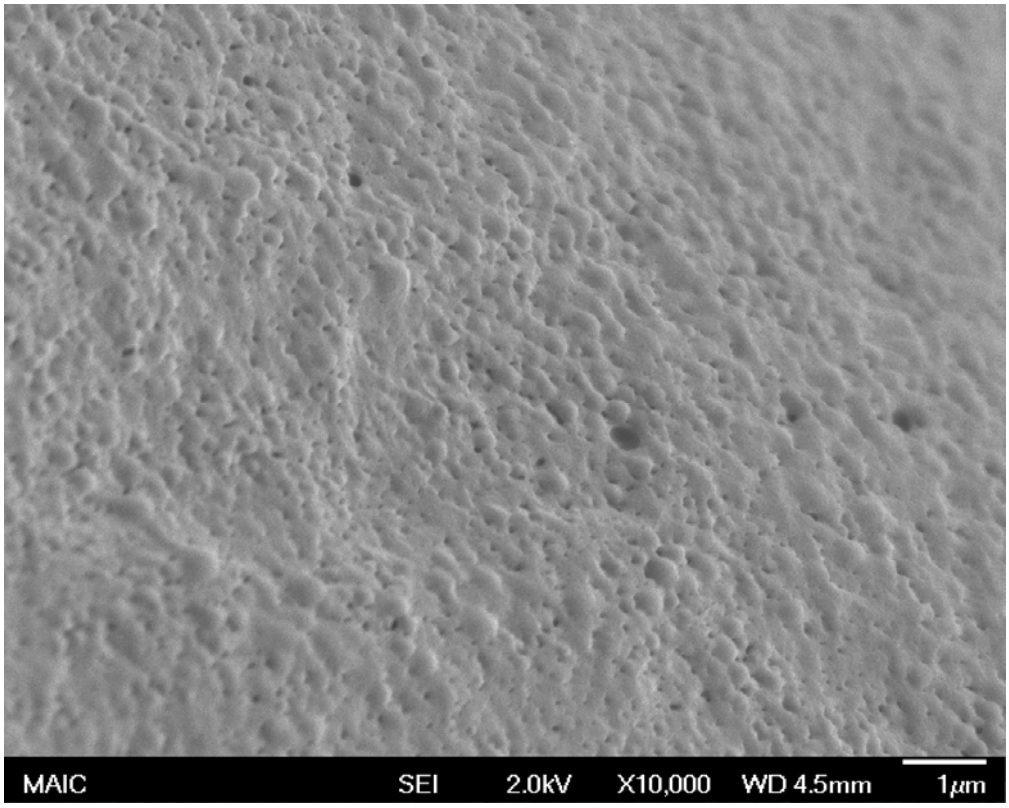


Figure 2-2. Scanning electron microscopy image of triamcinolone acetonide loaded 150 nm nanoparticles. Bar indicates 1 μm

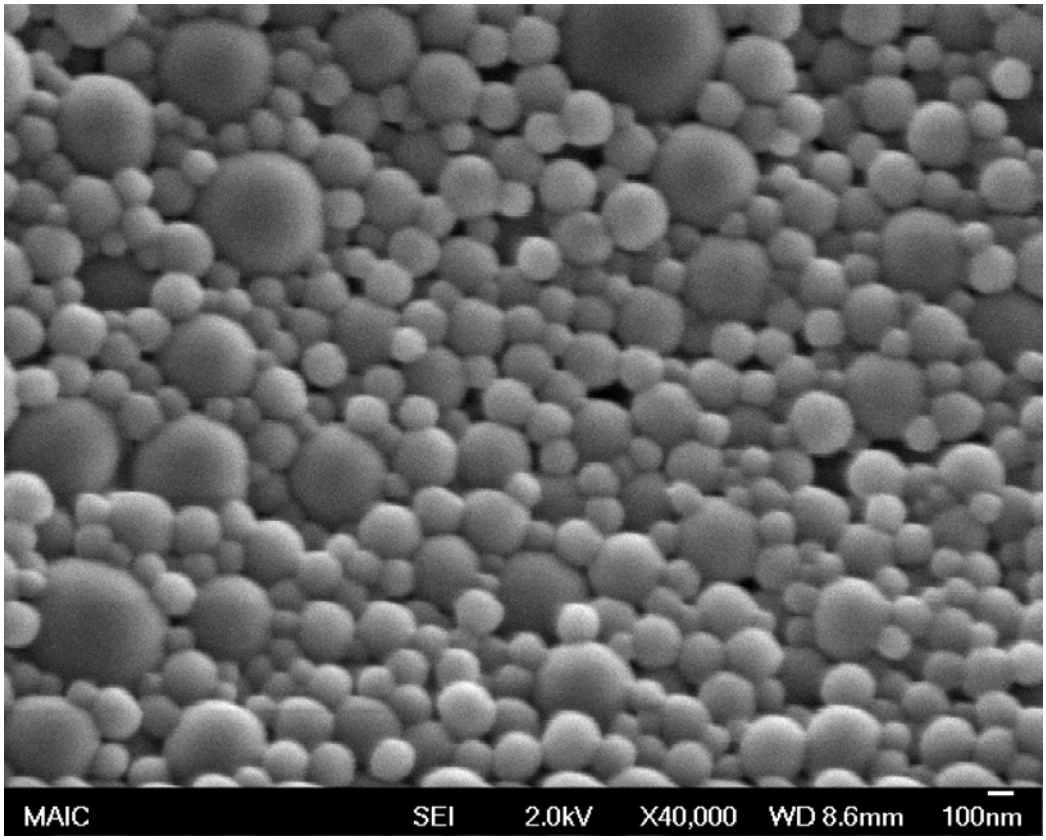


Figure 2-3. Scanning electron microscopy image of triamcinolone acetonide loaded poly(lactide-co-glycolide) 415 nm nanoparticles. Bar indicates 100 nm

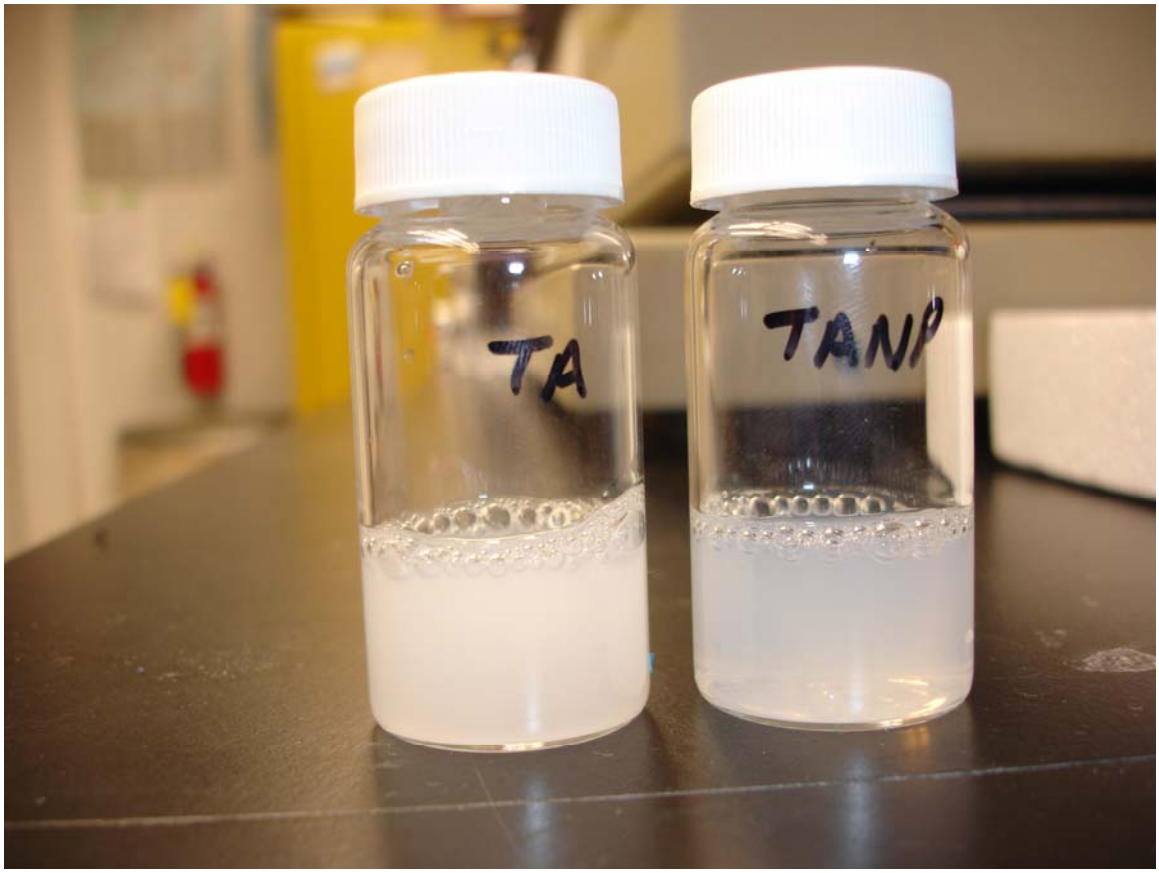
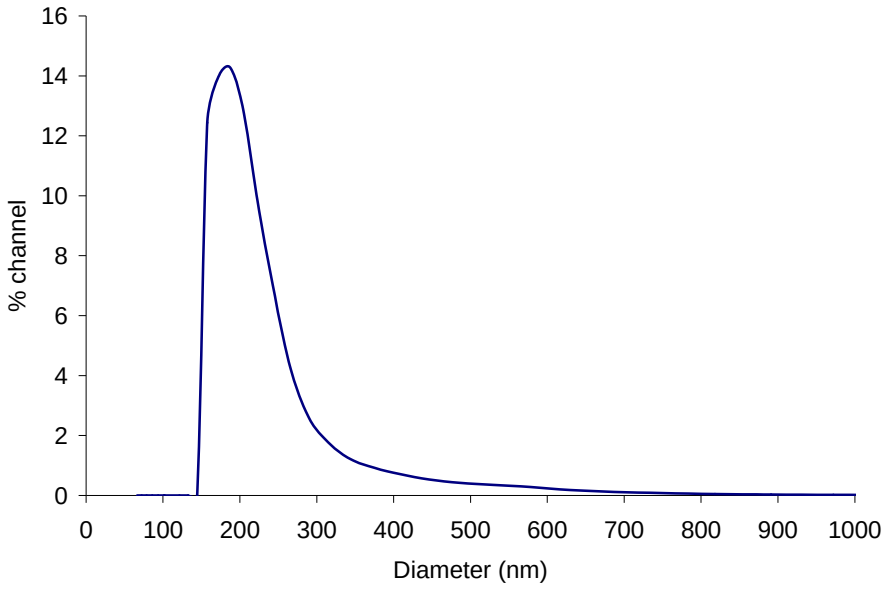
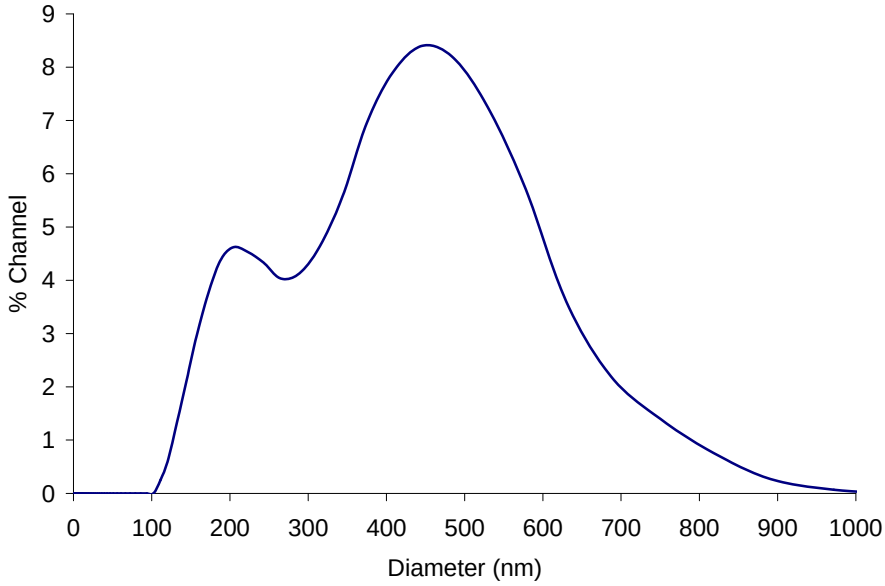


Figure 2-4. Turbidity of micronized triamcinolone acetonide and triamcinolone acetonide nanoparticles prepared with 1:10 ratio



A



B

Figure 2-5. Particle size distribution (A) 172 nm (B) 415 nm

Table 2-1. Effect of polymer concentration on particle size and loading

Formulation	PVA	Drug:Polymer	Size (nm)	Drug Loading (%wt/wt)	EE%
F1	1%	1:5	115 ±13*	12.7 ± 0.9*	48.36 ± 3.6*
F2	1%	1:10	172 ± 12	8.9 ± 0.5	61.64 ± 3.84
F3	1%	1:20	200 ±7	5.3 ± 0.001*	65.51 ± 2.30
F4**	1%	1:10	418± 22*	9.02 ± 0.17	78.8 ± 1.529*

*Statistically significant compared to F2 if $P < 0.05$. **400mg of polymer was used

Table 2-2. Effect of drug concentration on particle size and loading

Formulation	Drug (mg)	Drug:Polymer	Size (nm)	Drug Loading (%wt/wt)	EE%
F5	5	1:10	161 ± 4	3.8 ± 0.1*	20.19 ± 0.1*
F2	10	1:10	172 ± 12	8.9 ± 0.5	61.004 ± 2.2
F6	15	1:10	153 ± 4	10.5 ± 1.1*	78.6 ± 0.9*

* Statistically significant compared to F2 if $P < 0.05$

Table 2-3. Effect of surfactant (PVA) concentration on particle size and loading

Formulation	PVA	Drug:Polymer	Size (nm)	Drug Loading (%wt/wt)	EE%
F2	1%	1:10	172 ± 12	8.9 ± 0.5	61.0 ± 2.2
F7	4%	1:10	120 ± 30*	4.2 ± 0.4*	20.0 ± 3.0*
F8**	4%	1:10	108 ± 9*	7.9 ± 1.2	41.0 ± 6.9*

*Statistically significant compared to F2 if $P < 0.05$. ** shows 4% formulation where the aqueous phase was saturated with the drug

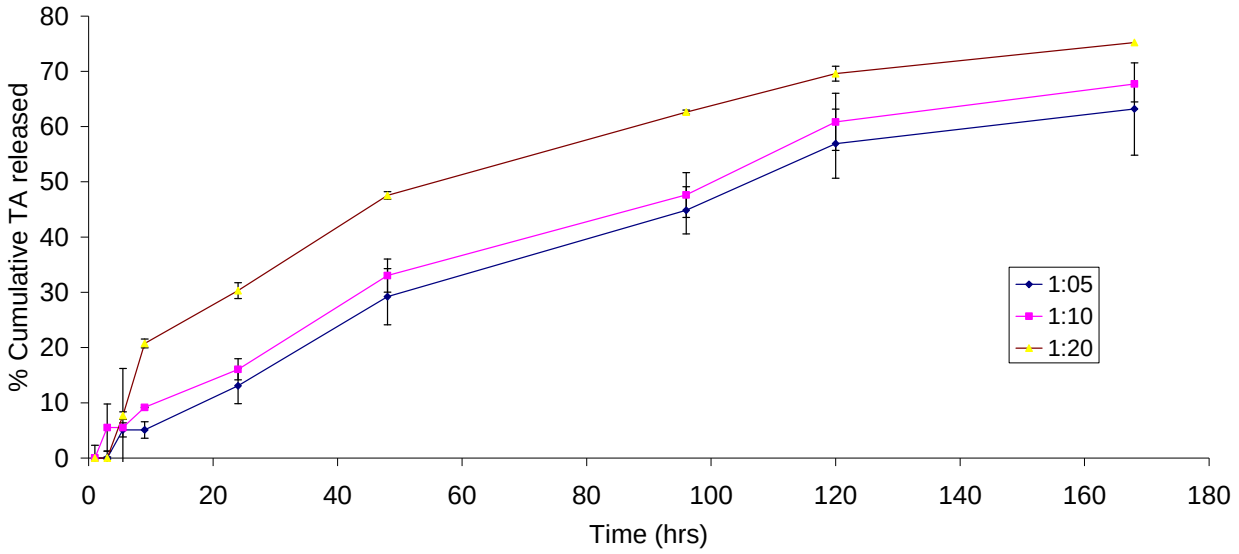


Figure 2-6. Effect of polymer concentration on *in vitro* cumulative triamcinolone released from nanoparticles. All the studies were performed at 37°C. Data are expressed as the mean ± SD.

Table 2-4. Zero order and Higuchi equations of *in vitro* drug release showing the effect of polymer

Formulation	Zero Order		Higuchi Equation	
1:5	R^2 = 0.9818	Y=0.4305X + 5.0135	R^2 = 0.9894	Y= 6.8924X +20.12
1:10	R^2 = 0.9751	Y= 0.4341X + 8.1279	R^2 = 0.9827	Y= 6.951X + 17.23
1:15	R^2 = 0.9618	Y= 0.3902X + 24.416	R^2 = 0.9881	Y= 6.305X +1.1349

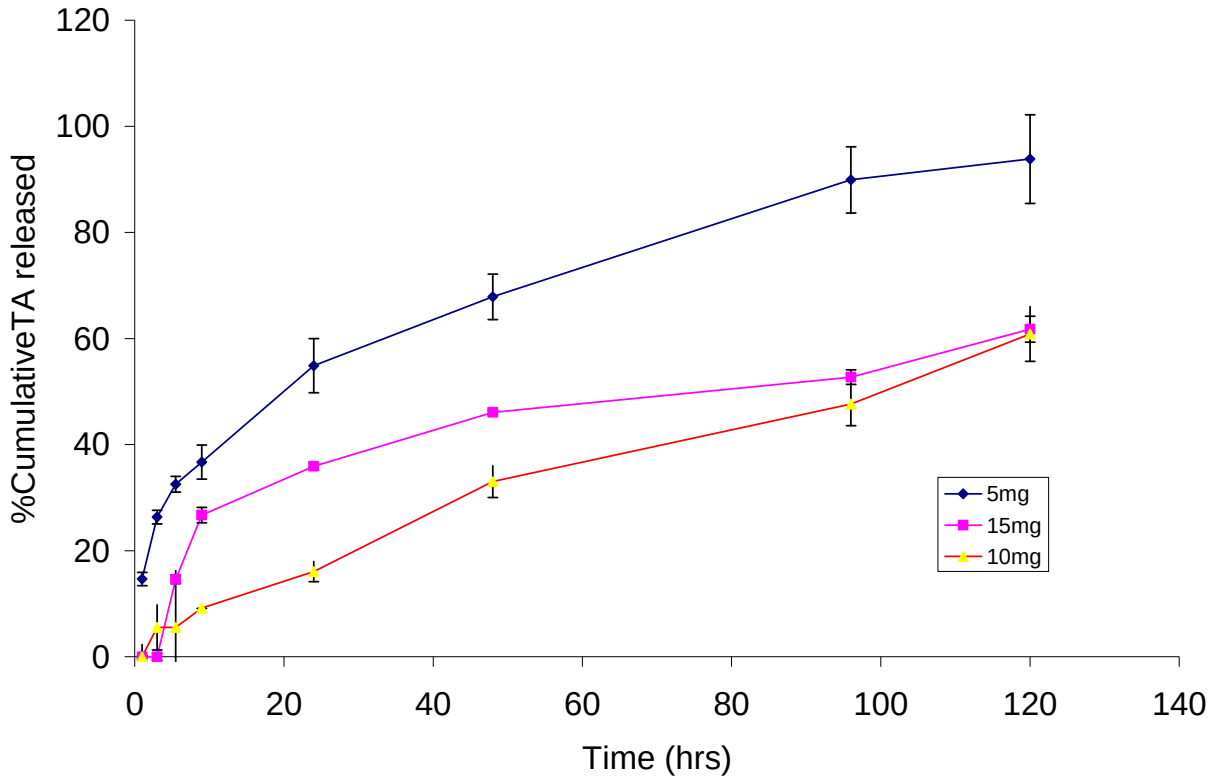


Figure 2-7. Effect of drug concentration on *in vitro* cumulative triamcinolone released from nanoparticles. All the studies were performed at 37°C. Data are expressed as the mean ± SD.

Table 2-5. Zero order and Higuchi equations of *in vitro* drug release showing the effect of drug

Formulation	Zero Order		Higuchi Equation	
5 mg drug	R^2 = 0.9967	Y= 0.4247X + 63.901	R^2 = 0.9973	Y= 6.7759X + 39.392
10 mg drug	R^2 = 0.9751	Y= 0.4341X + 8.128.	R^2 = 0.9827	Y= 6.951X + 17.230
15 mg drug	R^2 = 0.9551	Y=0.2434X + 31.584	R^2 = 0.9705	Y= 3.8926X + 17.402

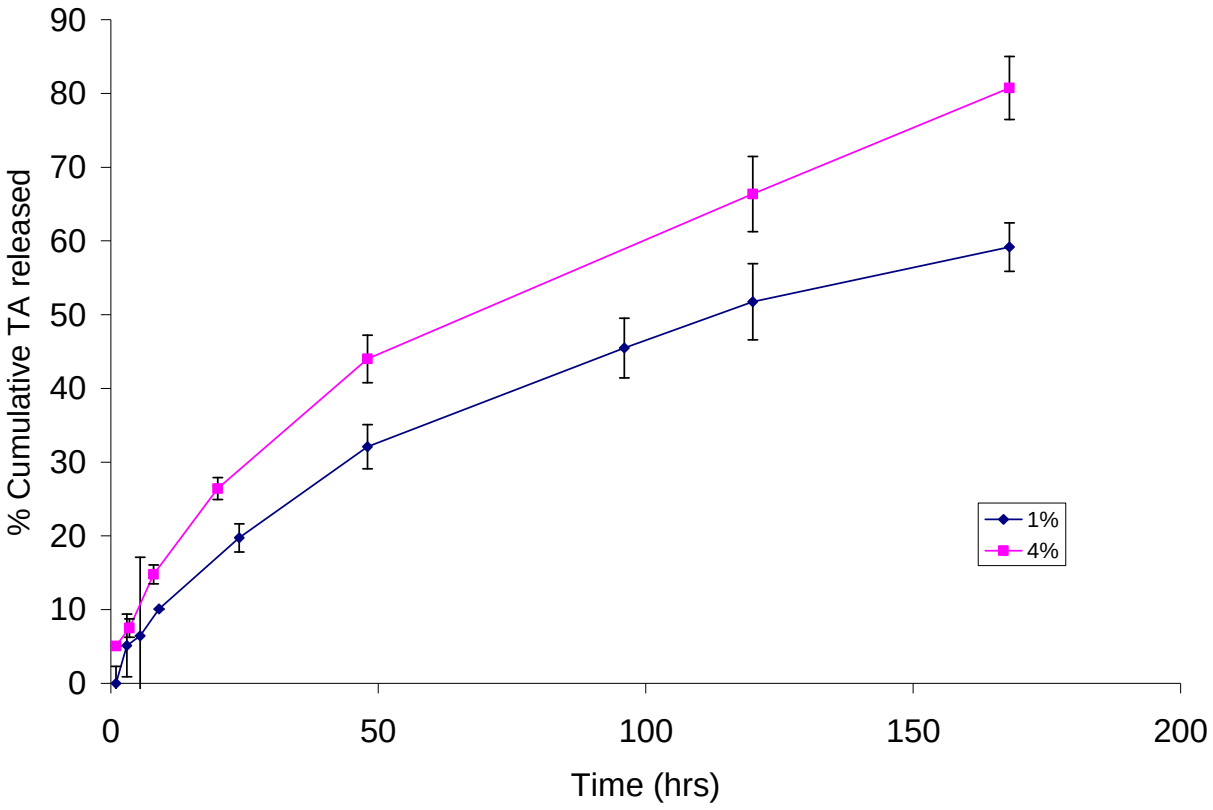


Figure 2-8. Effect of surfactant poly(vinyl alcohol) concentration on *in vitro* cumulative triamcinolone released from nanoparticles. All the studies were performed at 37°C. Data are expressed as the mean ± SD.

Table 2-6. Zero order and Higuchi equations of *in vitro* drug release showing the effect of surfactant

Formulation	Zero Order		Higuchi Equation	
1% PVA	R^2 = 0.97510	Y= 0.4341X + 8.1279	R^2 = 0.9827	Y= 6.951X + 17.23
4% PVA	R^2 = 0.09892	Y= 0.467X + 19.106	R^2 = 1	Y= 7.4013X -. 6.7794

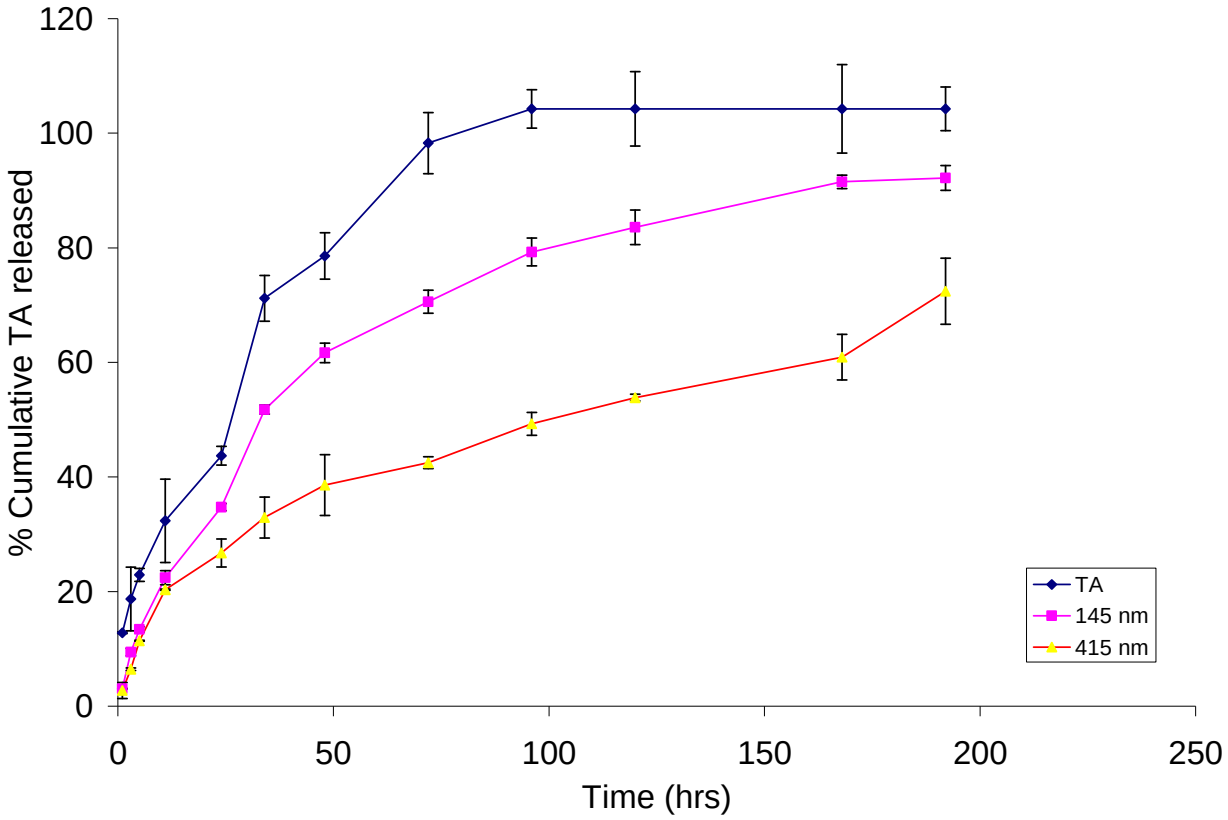


Figure 2-9. Effect of size of the nanoparticles on *in vitro* cumulative triamcinolone released from PLGA particles. All the studies were performed at 37°C. Data are expressed as the mean ± SD.

Table 2-7. Zero order and Higuchi equations of *in vitro* drug release showing the effect of micronized triamcinolone acetonide (TA), smaller nanoparticles (NP) and larger nanoparticles

Formulation	Zero Order		Higuchi Equation	
TA	R^2 = 0.7395	Y= 0.4899X+ 34.699	R^2 = 0.9007	Y= 8.116X+10.87
Smaller NP	R^2 = 0.8313	Y= 0.4592X +21.519.	R^2 = 0.9667	Y= 7.2552X + 1.2001
Larger NP	R^2 = 0.9042	Y=0.3184X+ 14.315	R^2 = 0.9882	Y= 5.0435X +0.513

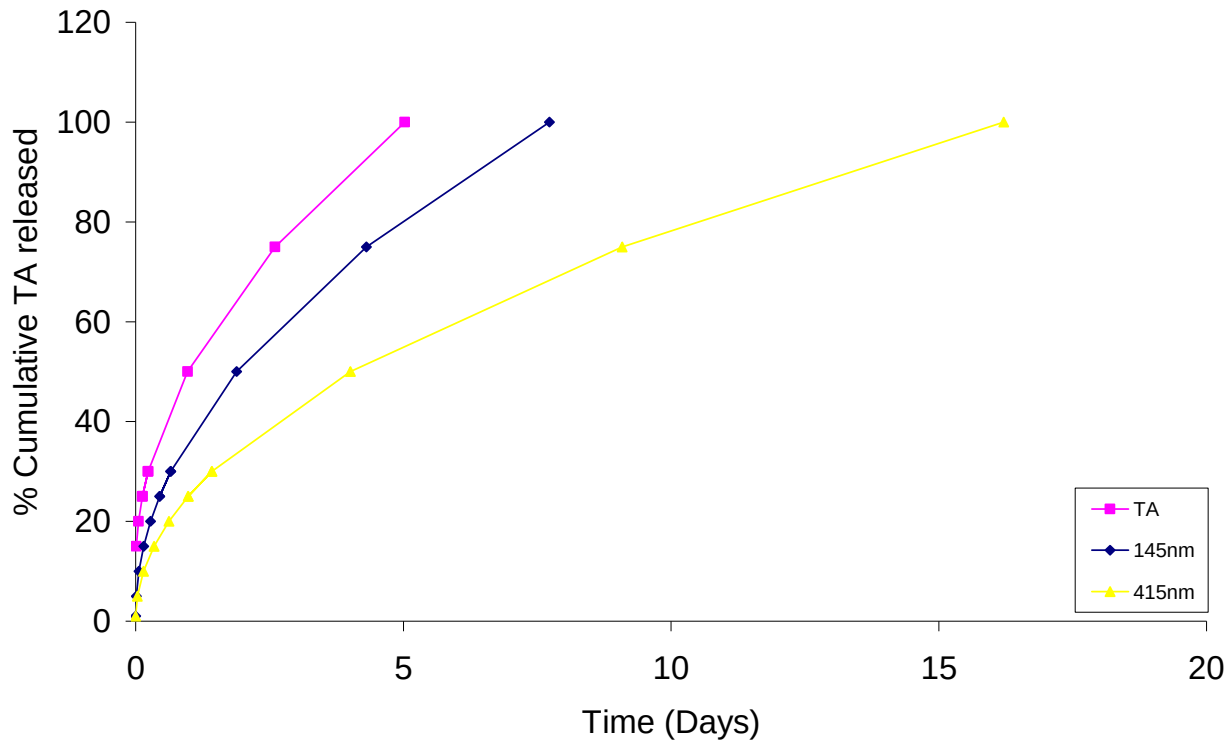


Figure 2-10. Results of *in vitro* release studies extrapolated to 100% cumulative drug release (A) micronized TA (B) smaller TA nanoparticles (C) larger TA nanoparticles

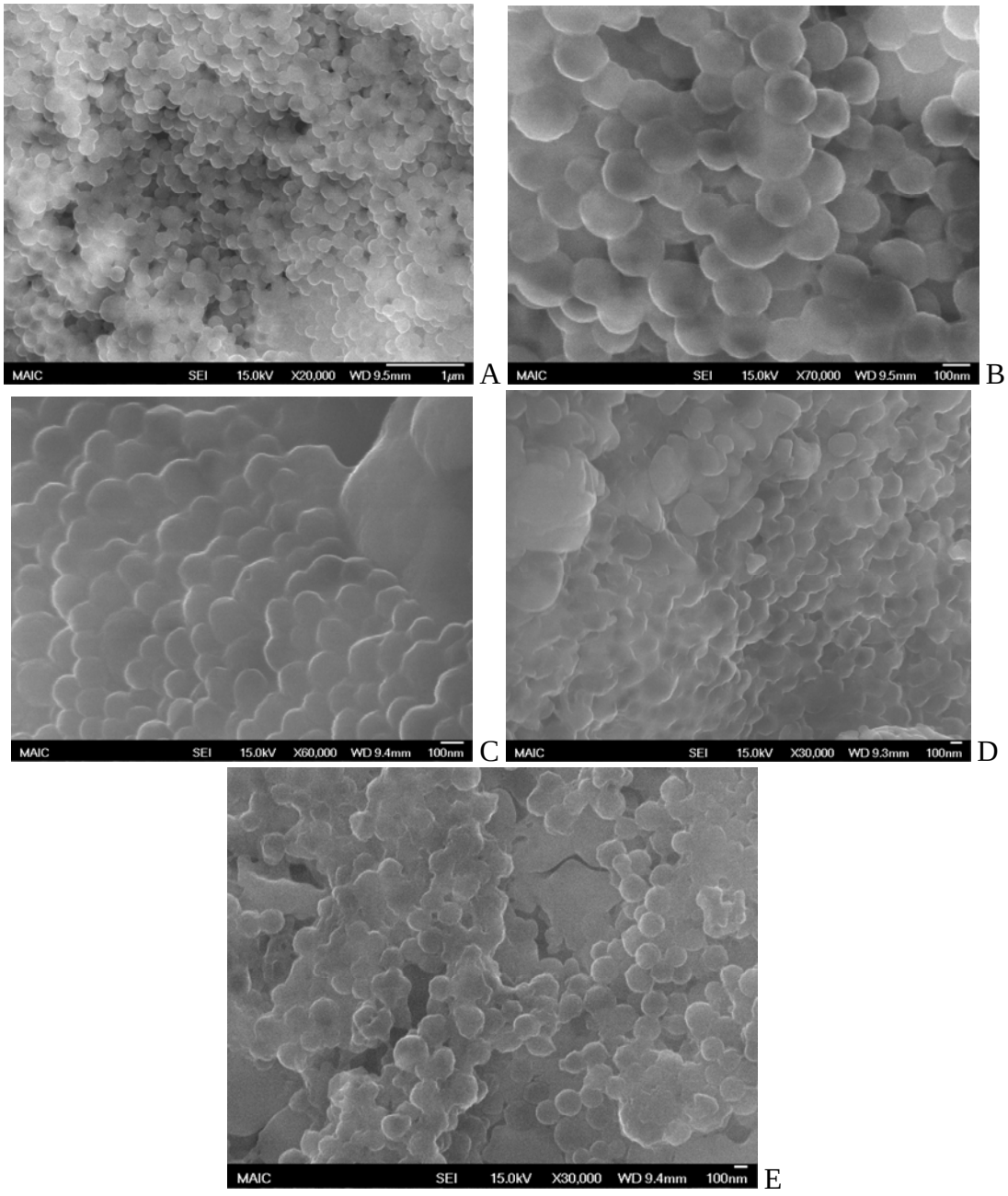


Figure 2-11. Scanning electron microscopy images taken during *in vitro* release studies (A). day 0 (B) day 1 (C) day 5 (D) day 10 (E) day 15

Table 2-8. Relationship between various parameters to control the particle size and drug content for nanoparticles prepared by emulsification-solvent evaporation method

Nanoparticle characteristics	Effect of formulation parameters
Effect on particle size	Increase in polymer concentration in organic phase increased the size of the particle Decrease in surfactant concentration increased the size of the particles Increasing the energy during emulsification decreased the size of the particle The drug concentration did not show effect on particle size
Effect on drug loading	Increase in drug concentration increased drug loading Decrease in drug loading was seen with increase in surfactant concentration Increase in particle size increased drug loading Saturation of the aqueous phase with drug increased drug loading.

CHAPTER 3
EFFECT OF TRIAMCINOLONE ACETONIDE AND ITS NANOPARTICLES ON RETINAL PIGMENT EPITHELIAL CELLS

Introduction

Retinal pigment epithelium is a major layer within the retina that separates the retina from the remaining posterior ocular tissue layers. Retinal pigment epithelial (RPE) cells express vascular endothelial growth factor (VEGF), which plays a critical role in the development of various posterior ocular conditions. VEGF, an angiogenic mitogen has been implicated in various vascular diseases in the eye including choroidal neovascularization, macular edema and diabetic retinopathy. Elevated levels of VEGF were reported in patients suffering from diabetic retinopathy and choroidal neovascularization [15]. Hence, agents that can inhibit VEGF secretion offer an attractive therapeutic modality.

Most of the research has been on macromolecules such as VEGF antibodies [94] and VEGF aptamers , which have poor stability and permeability [17]. Corticosteroids are gaining interest recently due to their angiogenic and antipermeable properties. These properties are in part due to their ability to inhibit VEGF secretion. Previous studies showed corticosteroids could inhibit VEGF secretion in airway and alveolar epithelial cells [95].

Intravitreal delivery is an effective route to deliver drugs to the posterior region of the eye. It helps to overcome the various ocular barriers and the long diffusional distance required for attaining therapeutic concentration for posterior ocular conditions. As repeated injections are not desirable, large doses of drug are needed. These high concentrations may be toxic to ocular cells. Researchers have shown the toxicity of triamcinolone on ARPE-19 cells [96]. Retinal pigment epithelium plays a crucial role in maintaining the integrity of the outer retina blood barrier and supporting normal function of the retina. Since the retinal pigment epithelium is in close proximity to the vitreous space, separated from it only by the neuroretina, any damage to this

monolayer poses the risk of its cytotoxicity and retinal dysfunction. Hence, particulate systems such as microparticles, nanoparticles and liposomes could be used for site-specific and sustained delivery of drugs [96-98]. Such delivery systems will likely modify the disposition of drugs at the site of RPE cells, sustain drug release, reduce toxicity and increase intracellular levels of the drugs in RPE. We hypothesized that nanoparticles can decrease cytotoxicity compared to the free drug and also sustain drug release.

Cellular uptake is an important aspect of nanoparticle delivery. Because retinal pigment epithelial cells *in vivo* phagocytose particles [99], sustained high intracellular drug levels can likely be achieved by preparing particulate drug delivery systems that can be injected into the vitreous. Previous studies demonstrate that uptake and retention of drug carriers like nanoparticles are affected by cellular processes such as endocytosis and exocytosis. Studies have shown that nanoparticles formulated using polymers such as poly(lactide-co-glycolide), are taken up into cells through active process such as endocytosis [100]. Although it is well-known that retinal pigment epithelial cells take up particles, the factors affecting the uptake are not well characterized [101, 102]. Understanding the factors influencing particle uptake would help in developing a suitable particulate system for drug delivery to the retinal pigment epithelium.

Human ARPE-19 cells have morphological and functional properties similar to retinal pigment epithelial (RPE) cells *in vivo* [95]. They are capable of differentiating and proliferating similar to the RPE cells. In our studies we use ARPE-19 cells to show the effect of corticosteroids and their nanoparticles on ocular cells.

The overall objectives of these studies are as follows,

- determine the effect of triamcinolone acetonide on VEGF secretion in ARPE-19 cells
- determine the toxicity of micronized triamcinolone acetonide compared to its nanoparticles on retinal pigment epithelial cells and

- determine the mechanism of uptake of triamcinolone acetonide nanoparticles in ARPE-19 cells.

Materials and Methods

Materials: ARPE -19 cells, culture medium Dulbecco's modified Eagle's medium Ham's F-12 [DMEM-F12], fetal bovine serum (FBS), penicillin-streptomycin, trypsin were obtained from Gibco (Carlsbad, CA). The cells were cultured either in cell culture flasks T-75 cm^2 and the experiments were conducted in 96-well plates or 24 well plates obtained from Fisher (Pittsburgh, PA). Cell toxicity kit and cell lysis reagent were obtained from Sigma (St. Louis, MO). VEGF ELISA kit was obtained from R&D systems (Minneapolis, MN). Pierce BCA protein assay kit (Rockford, IL) was used for protein analysis. Poly(lactide-co glycolide) was obtained from Lactel absorbable polymers (Pelham, AL). The HPLC grade methylene chloride and acetonitrile were obtained from Fisher Scientific.

Cell Culture

Human ARPE-19 cells were grown in 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's and Ham's F12 medium, containing 10% fetal bovine serum, and antibiotic mixtures of 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. The cells were cultured in 75 cm^2 flasks. The cultures were maintained in a humidified 5% CO_2 environment at 37°C. Cells grown in a 75 cm^2 T-flask are passed after they have reached 80-90% of confluence at a split ratio of 1:2. The medium was changed every 2 days.

For cell splitting, the old media in the flask is discarded and the cell layer is briefly rinsed with PBS. Trypsin 5 ml is added and incubated for 10min. Later 5 ml of the media was added into the flask and the cell suspension is transferred into a centrifuge tube and spun at 125 g for 5 minutes. The supernatant is then discarded and cells are re-suspended in fresh growth media. Appropriate aliquots of cell suspension are transferred to new flasks and incubated at 37°C.

Vascular Endothelial Growth Factor Secretion

Cells of ARPE-19 cultured in the tissue flask are trypsinised and seeded onto a 24 well plate and allowed to grow to confluence. The media was replaced every two days. On the day of the study media was replaced with 1% FBS media and allowed to remain in quiescence for 12 hours. After the quiescence period, the monolayers are incubated with triamcinolone acetonide (1, 10 and 100 µM). The culture media was collected at the end of 12 hours. The secreted VEGF in supernatants is quantified by ELISA method capable of detecting VEGF 165 and the cell protein content was assayed using the BCA protein assay kit after lysing the cells and the VEGF secretion was normalized to total protein.

Cell Toxicity

Cells of ARPE-19 were seeded at a density of 10,000 cells/well and were allowed to attach overnight in 96-well plates. The cells were then exposed to corticosteroids or their nanoparticles (0, 1, 10, 100 and1000 µM). At the end of 24 hour incubation, the cells were incubated with 25 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) for 3 hours at 37°C. Viable cells will reduce MTT to formazan. Finally 100 µl of isopropanol was added to dissolve formazan crystals and the absorbance of this solution was measured at a wavelength of 540 nm using a micro-plate reader. The cell viability is then determined by the relative formazan formation after corticosteroid and the nanoparticles treatments compared to the control group.

Cell Uptake

Human ARPE-19 cells were seeded in a 24-well plate at a density of 50,000 cells/well and allowed to attach overnight. Cells were then treated with nanoparticle suspension or equivalent dose of triamcinolone acetonide solution in complete growth medium. To determine the effect of dose of nanoparticles on uptake, cells were treated with various doses (50,100, 200 µg/ml) of

triamcinolone nanoparticles for 2 hours. To determine the effect of time of treatment, cells were treated with constant dose (100 µg/ml) of triamcinolone nanoparticles for varying periods of time (0.5, 1, 2 and 4 hours). To study the effect of temperature on cellular uptake of nanoparticles, cells were preincubated at 4°C for 1 hour and then treated with the nanoparticle suspension (100 µg/ml) at 4°C for 2 hours.

At the end of the treatment period, the cell monolayer was washed three times with ice cold PBS. Cells were then lysed using 150 µl of cell lysis reagent. The protein content of the cell lysate was determined using the Pierce protein assay reagents. The cell lysates were then analyzed for corticosteroid content. Cell lysates were mixed with 300 µl of methanol and incubated at 37°C for 6 hours on a shaker. The samples were centrifuged at 13,000 rpm for 10 minutes. Supernatants were then analyzed for corticosteroids using HPLC. The drugs were separated with a C-18 column. The mobile phase for the assay consisted of acetonitrile and acetate buffer mixture (50:50 vol/vol). The flow rate was 1 ml/min. A standard plot was constructed for corticosteroids in cell lysate reagent under identical conditions that was used for the sample preparation. Data was expressed as corticosteroid accumulation normalized to total cell protein. The calibration curve was plotted with standards from 0.5 to 20 µg/ml. ($r^2 > 0.99$).

Statistical Analysis

All statistical analyses, one way ANOVA and t-test were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) with p value less than 0.05 considered as significant. The effect of VEGF secretion and effect of cellular uptake on time and concentration were analyzed using ANOVA. T-test was used to test the energy dependence on cellular uptake and the MTT assay results.

Results

Vascular Endothelial Growth Factor Secretion

Effect of triamcinolone acetonide on VEGF secretion was studied using ARPE-19 cells. The cells were initially treated with 1, 10 and100 μM concentrations of triamcinolone acetonide and the VEGF secretion at the end of 12 hours was measured. Figure 3-1 shows the measured concentrations of VEGF secretion. Compared with no treatment (control), triamcinolone acetonide at 1, 10, 100 μM reduces the VEGF secretion by 60%, 51% and 54 %, respectively.

Cell Toxicity

Cell toxicity of corticosteroids and their nanoparticles was studied to determine their safety.

Results are expressed as units of absorbance of MTT at 540 nm. The absorbance readings under different treatments are further converted to relative percent cell viability for comparative analysis. The percent viability in the presence of triamcinolone acetonide was calculated by dividing the absorbance reading of cells under different concentrations of corticosteroids by the absorbance reading of cells under normal growth (assumed 100% viability) in the absence of drugs.

Experiments showed that triamcinolone acetonide causes a significant reduction in cell numbers as long as the cells had been exposed for more than 24 hours. Further, at 24 hours time point triamcinolone showed toxicity above 10 μM. Figure 3-2 shows the effect of triamcinolone concentration on cell viability after 24 hours. The results show that the cell viability decreased from 100% for control to 74, 86 and 89% with 1000, 100, 10 μM of free triamcinolone acetonide, respectively.

Figure 3-3 shows the effect of triamcinolone, triamcinolone nanoparticles and blank nanoparticles on cell viability at 24 hour point at various levels of drug concentration. It was apparent that the cytotoxic effects of triamcinolone was concentration-dependent. Decrease in toxicity was seen with triamcinolone nanoparticles compared to the free drug.

Cell Uptake

Cellular uptake of triamcinolone acetonide nanoparticles as nanoparticles or free drug was studied in ARPE-19 cells. Figure 3-4 shows the cell uptake of free triamcinolone acetonide micronized compared with its nanoparticles. The results show that nanoparticle formulation of triamcinolone acetonide has similar uptake compared to free drug.

Kinetics of cellular accumulation of corticosteroid nanoparticles is studied by the effect of concentration, time and temperature on cellular accumulation. Figure 3-5 shows the effect of dose on cell uptake. At 50, 100 and 200 µg/ml dose, cellular accumulation of 149, 352 and 748 µg/mg protein was observed. This indicates an increase in uptake with increase in dose.

Figure 3-6 shows the effect of time on cell uptake when the drug concentration was maintained at 100 µg/ml. At 0.5, 1, 2 and 4 hours, the nanoparticles accumulation levels of 90, 305, 352 and 612 µg/mg was observed. This indicates an increase in uptake with time.

Finally the energy dependence of nanoparticles uptake in cells was studied by comparing the uptake of cells by incubating at 37°C and 4°C. Figure 3-4 shows the observed results. Decreasing active processes in cells by incubating cells at 4°C decreased the nanoparticles uptake approximately 5times. Figure 3-7 shows the effect of temperature on the cell uptake of nanoparticles.

Discussion

Mechanism of action for angiostatic and antipermeable property of corticosteroids is not completely known. One of the reasons for their angiostatic and antipermeable properties might be due to their ability to inhibit VEGF secretion. VEGF is responsible for causing neovascularization and edema. The study was conducted in the presence of drug alone to determine the VEGF inhibitory property of triamcinolone acetonide. Our results show that triamcinolone acetonide was able to inhibit VEGF secretion in ARPE-19. This is consistent with the finding that corticosteroids reduce VEGF in cultured aortic vascular smooth muscle cells [103] and also can reduce VEGF mRNA and protein expression in cultured eosinophils [46]. Counter effects of corticosteroids on VEGF have also been demonstrated in a recent rabbit study, in which intravitreal VEGF injections caused a time and dose-dependent breakdown of blood-retina and blood-aqueous barriers and led to vascular leakage. The breakdowns were blocked both by dexamethasone and triamcinolone acetonide, but not the nonsteroidal anti-inflammatory drug (NSAID) indomethacin.

Retinal pigment epithelium is separated from the vitreous only by the presence of neuroretina. Therapeutic concentration to the posterior region of the eye can be achieved through intravitreal delivery. The drug delivered to the vitreous are at high dose and can be toxic. The cell toxicity study serves as a tool to demonstrate the safety of corticosteroids and their nanoparticles on cells in the posterior region of the eye. Narayana et al. showed that triamcinolone acetonide caused a significant decrease in cell viability at concentrations of 100 and 200 µg/ml at all the three time points of 2, 6 and 24 hours. Similar results were also seen by Yeung et al. from 0.01 to 1 mg/ml concentration [46, 104]. Drug delivery systems such as nanoparticles help in attenuating adverse effects by sustaining the drug release without huge fluctuation in the peak concentration. A decrease in cell toxicity with nanoparticles compared

with the free drug was seen. The use of nanoparticles is an effective way to overcome the adverse affects associated with triamcinolone acetonide which is currently used in clinics. Though the exact mechanism of toxicity is not known, it has been reported that the glucocorticoid retinal cells cytotoxicity *in vitro* is mediated through alterations of mitochondrial activity.

In our study a slow increase in triamcinolone cellular uptake was seen with increase in concentration and time of incubation of the cells with the nanoparticles. The uptake of drug into the cells could be either due to the slow release of the drug from the nanoparticles into the media, which is then taken up by the cells or due to the direct uptake of the nanoparticles by the ARPE-19 cells, which then release the drug in the cells. The cells were washed three times with PBS to remove any free drug in the cells. Uptake of the nanoparticles into the cells was considered as the nanoparticles used in our study were below 200 nm and nanoparticles of size below 200 nm have been reported to accumulate with in various cells including ARPE-19 cells [105]. Indicating that nanoparticles are taken up and concentrate within the cells.

Decrease in cellular uptake was also seen at lower temperature which indicates energy dependence of the cellular uptake of the particles. Although to confirm this we have not shown the decrease in uptake in the presence of metabolic inhibitors, Panyam et.al showed that uptake of PLGA nanoparticles in muscle cells depends on concentration, time and energy. They showed that the uptake was inhibited in the presence of metabolic inhibitors such as sodium azide [105].

Conclusion

Our studies show that triamcinolone acetonide inhibits VEGF secretion in retinal pigment epithelial cells. Cell culture studies in retinal pigment epithelial cells showed that triamcinolone acetonide loaded nanoparticles were less toxic compared to the micronized triamcinolone

acetonide alone. Nanoparticles were internalized efficiently by ARPE -19 cells and the uptake of nanoparticles was dose and time dependent. Triamcinolone acetonide nanoparticles formulated from poly(lactide-co-glycolide) polymer offer a nontoxic and efficient delivery system for the sustained intracellular delivery.

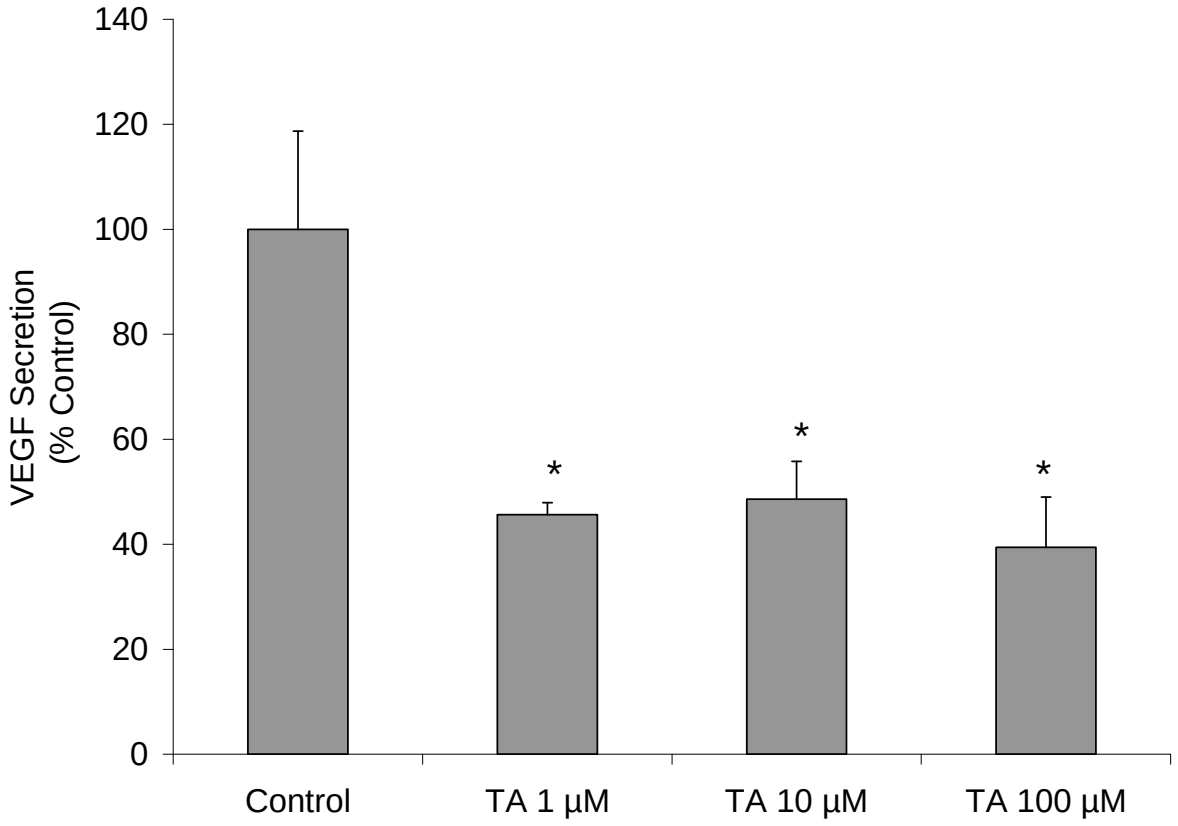


Figure 3-1. Triamcinolone acetonide inhibited secretion of VEGF in ARPE-19 cells. N=8.Data are expressed as the mean ± SD. *Significantly different from the control at $P < 0.05$.

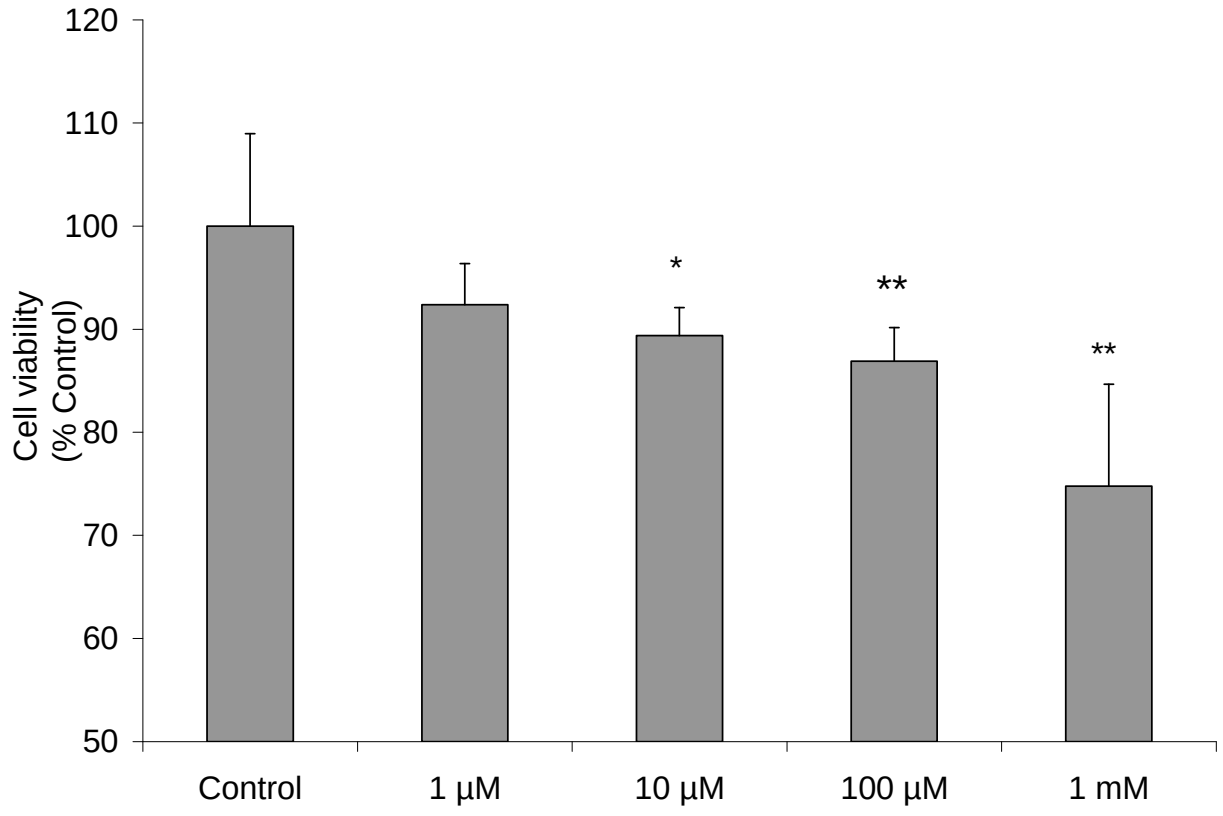


Figure 3-2. Viability of ARPE-19 cells after 24 hours with micronized triamcinolone acetonide. Treatments as determined by MTT assay. Data as mean ± SD (N=8). *Significantly different from the control at $P < 0.05$, **Significant $P < 0.01$.

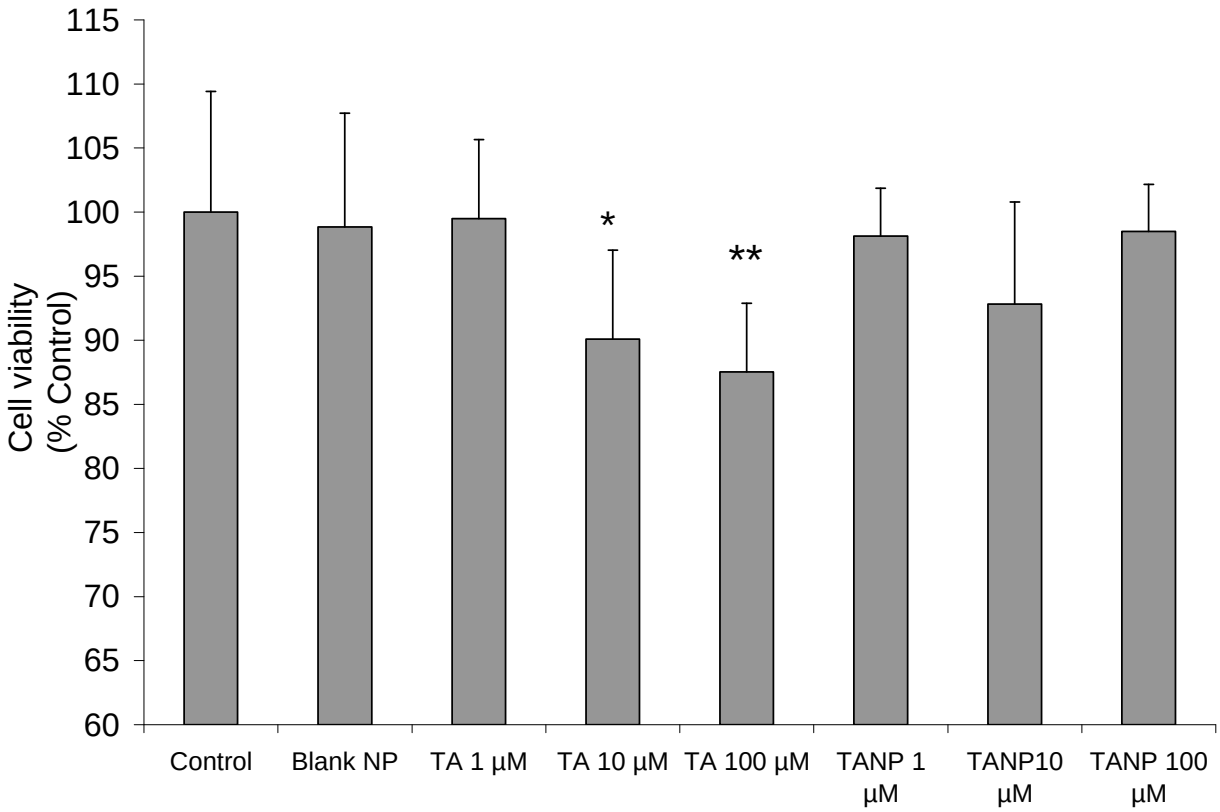


Figure 3-3. Viability of ARPE-19 cells after 24 hours with triamcinolone and triamcinolone nanoparticles. Treatments as determined by MTT assay. Data as mean ± SD (N=8). *Significantly different from the control at $P < 0.05$, **Significant $P < 0.01$.

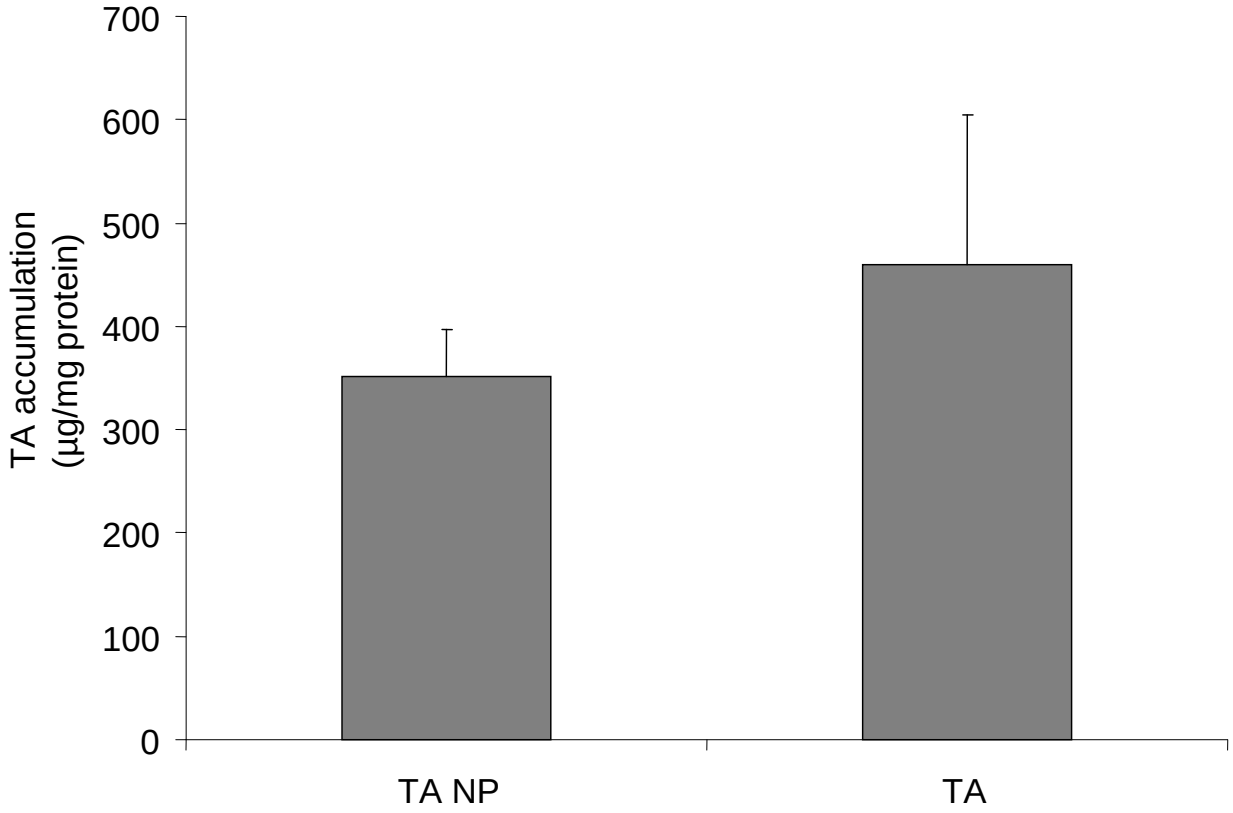


Figure 3-4. Cell uptake of micronized triamcinolone compared with its nanoparticles at 100 µg/ml at 37°C at the end of 2 hours. No statistically significant difference was seen between the two groups.

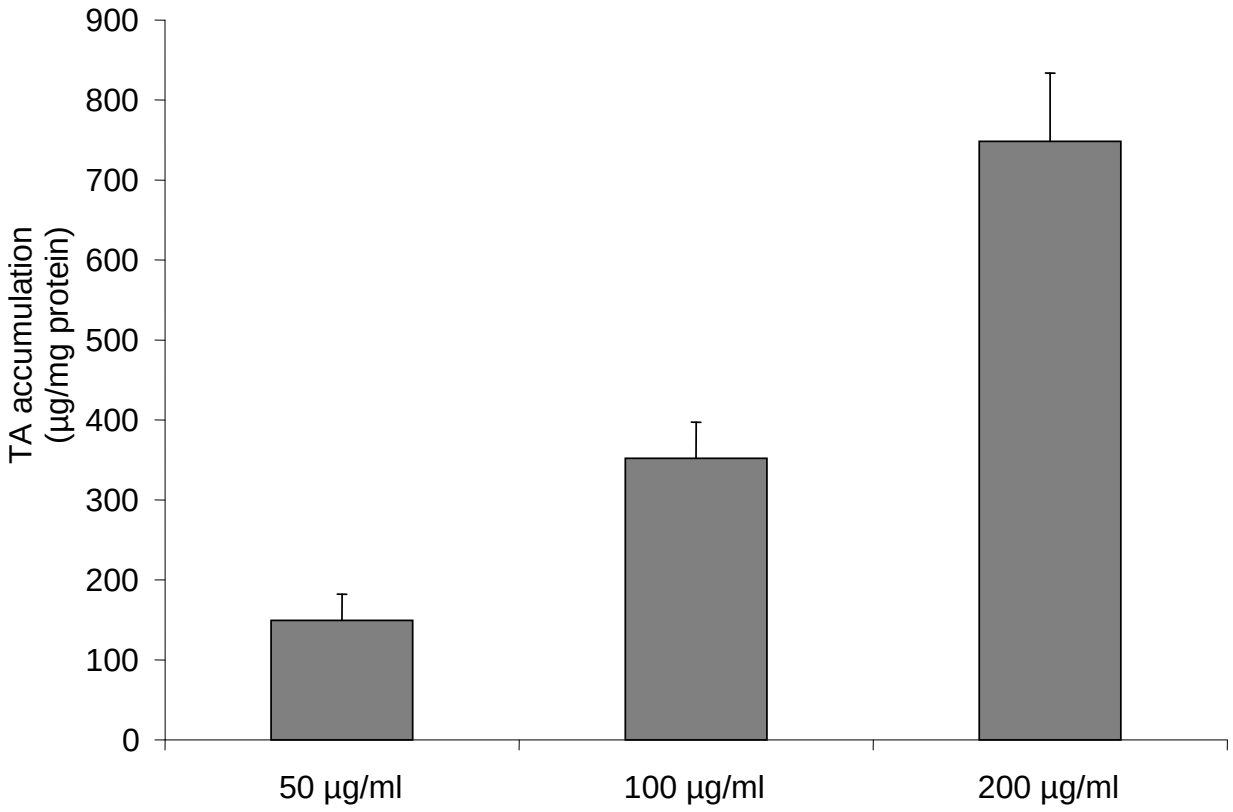


Figure 3-5. Effect of concentration on kinetics of nanoparticle uptake into ARPE-19 cells. Data as mean ± SD (N=4).

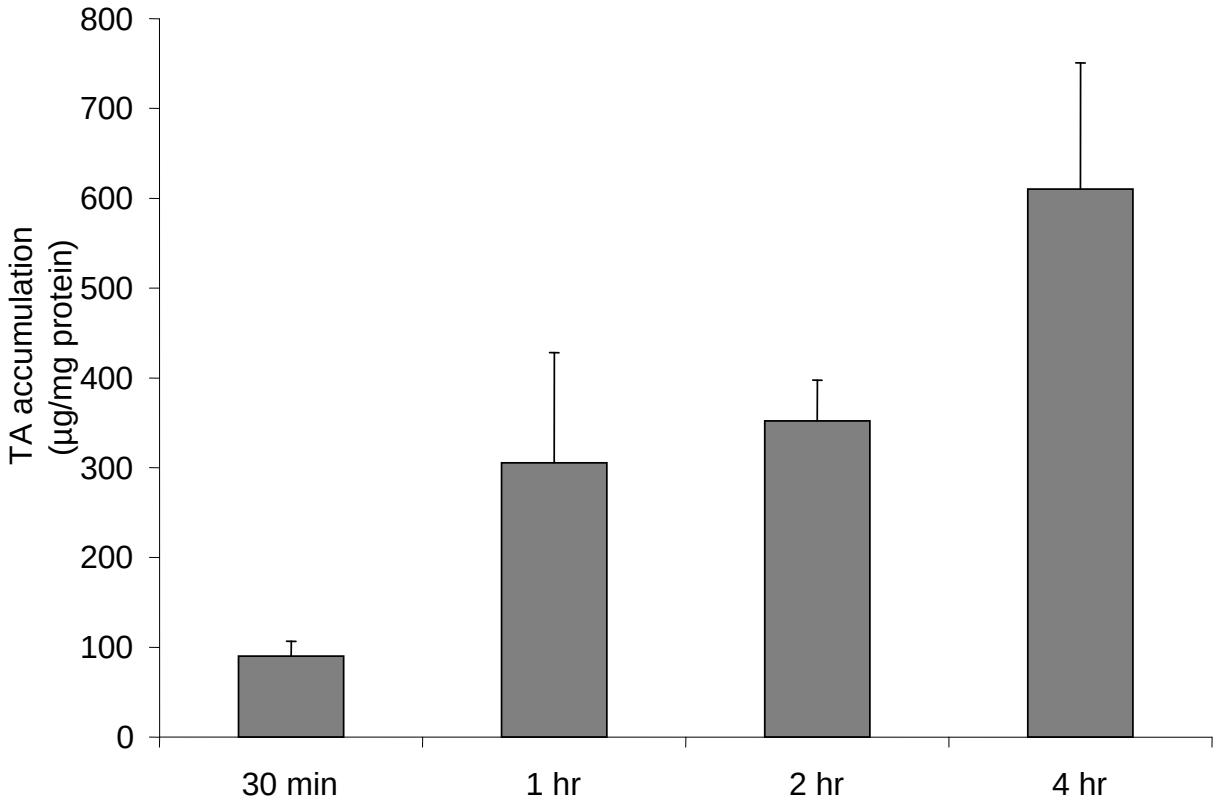


Figure 3-6. Effect of time on kinetics of nanoparticle uptake into ARPE-19 cells. Data as mean ± SD (N=4).

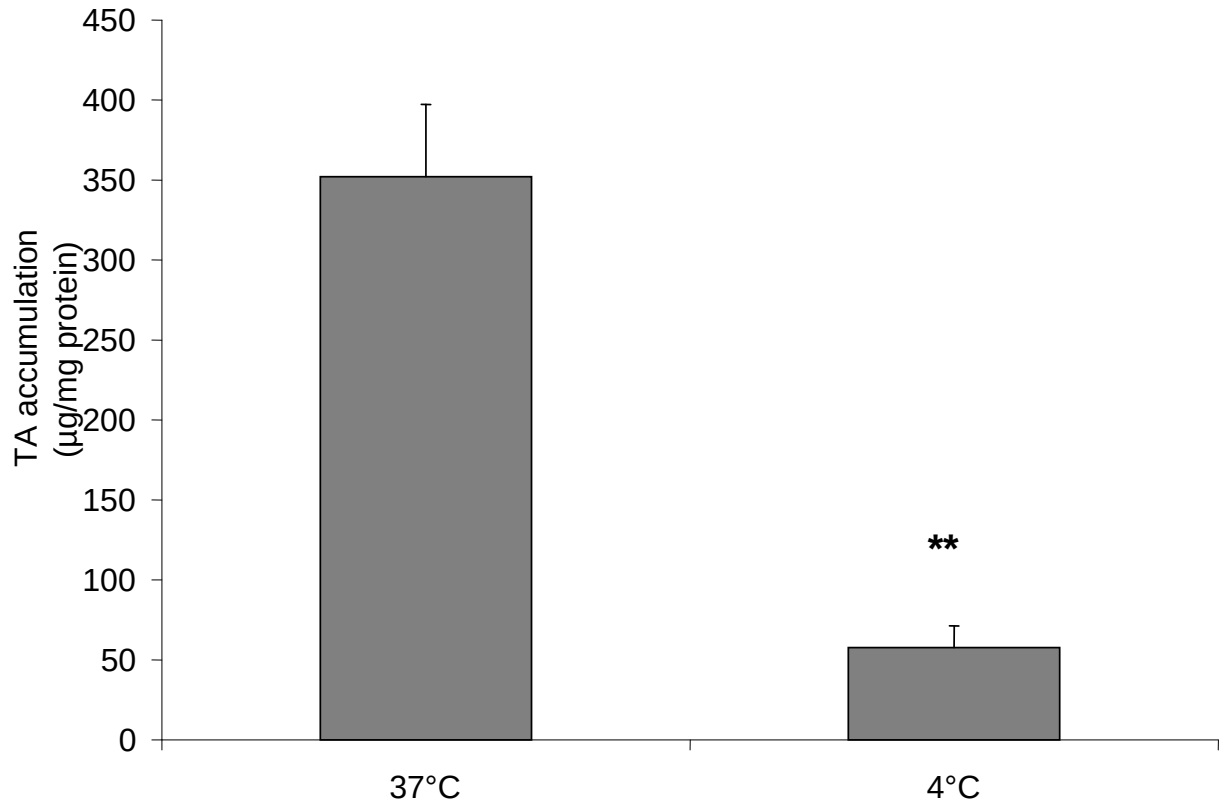


Figure 3-7. Effect of temperature on the kinetics of nanoparticle uptake into ARPE-19 cells. Data as mean ± SD (N=4). **Significantly different at $P < 0.01$.

CHAPTER 4
EFFECT OF INTRAVITREAL TRIAMCINOLONE ACETONIDE NANOPARTICLES ON LASER INDUSED CHOROIDAL NEOVASCULARIZATION IN MICE

Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness above the age of 55 in developed countries. However, the pathogenesis of age-related macular degeneration remains unclear; 4 to 10 million Americans are estimated to have some form of the disease. Severe loss of central vision frequently occurs with the exudative (wet) form of age related macular degeneration, as a result of the formation of a pathological choroidal neovascularization. Choroidal neovascularization contains abnormal blood vessels that leak fluid and blood. The leakage damages the structure and function of the retina especially at the macular region leading to loss of vision. Choroidal neovascularization is also generated in ocular histoplasmosis, high myopia, or some inflammatory diseases, after laser photocoagulation, and ocular trauma. The main treatments currently in use or under development for exudative age related macular degeneration include photodynamic therapy [106], submacular surgery, macular translocation [107], and transplantation [108]. However, most treated patients do not show visual improvement, and the surgical procedures can cause serious complications. The severe visual disability caused by this disease and the lack of an adequate treatment for the disease has lead to the search for new therapeutic strategies.

The recent resurgence in interest in choroidal neovascularization has been driven by the discovery of molecular mechanisms involved in this process and by the recognition that treatments targeting neovascularization will be a central strategy for treating the wet form of age-related macular degeneration. As the factors involved in the pathogenesis of choroidal neovascularization (CNV) are better understood, pharmacologic therapeutic agents are being explored. Antiangiogenic agents may be helpful in treating choroidal neovascularization without

destruction of the retina. Agents such as vascular endothelial growth factor (VEGF) inhibitors, matrix metalloproteinase inhibitors, and glucocorticoids are being considered to treat choroidal neovascularization as they can target the disease causing factors thus preventing reoccurrence. It may also prevent reoccurrence following laser treatment and might also be used as a prophylaxis[109]. Though VEGF inhibitors were shown to be effective in treating CNV, they are expensive and the systemic risks are yet to be investigated [110].

Inflammation and infiltration of inflammatory cells is associated with angiogenesis in biological systems [111]. Histopathological examination of excised CNV specimens shows increase in inflammatory cells. Also a relative increase in vascular endothelial growth factor which has been implicated in angiogenesis to the amount of inflammatory cell in excised specimens with CNV was shown [112]. Corticosteroids have antiangiogenic, antifibrotic, and antipermeable properties and have demonstrated to markedly inhibit corneal and retinal neovascularization rodents and primates [113].

Recently, nonrandomized and randomized case series have described intravitreal triamcinolone acetonide monotherapy for the treatment of choroidal neovascularization [114, 115]. In the majority of these reports, a short-term effect of improved retinal thickness, decreased neovascular exudation, and, in some cases, improved visual acuity was noted. However, over the long term, intravitreal corticosteroid injection monotherapy was largely not beneficial. This might be due the lack of a sustained effect. Holecamp and associates tried to address this problem by using the sustained-release nonbiodegradable flucinolone acetonide implant [116]. The surgical placement of the nonerodable implant carries potential surgical risks, including endophthalmitis, retinal tears or detachment, and vitreous hemorrhage [117].

Since choroidal neovascularization is one of the most afflictive problems facing ophthalmology, new solutions are needed. We hypothesized that nanoparticles of triamcinolone acetonide which are easy to administer via intravitreal injection are a suitable way to sustain drug delivery to overcome the progressive nature of choroidal neovascularization. In this study, we show that formation of a laser induced choroidal neovascularization can be prevented in a mouse model, by intravitreal injections of corticosteroid nanoparticles.

Materials and Methods

Laser Induced Choroidal Neovascularization

Mice were anesthetized with pentobarbital sodium (40 mg/kg) and pupils were dilated with compound tropicamide/phenylephrine. The 532 nm diode laser photocoagulation was delivered to the choroid of mice with 120 mW power for 100 msec duration, with a mean diameter of 100 μm, three burns to each eye. Treatment groups in the study included (n=5) blank nanoparticles, triamcinolone acetonide nanoparticles delivered through intravitreal route, triamcinolone acetonide nanoparticles delivered through intraperitoneal route and triamcinolone acetonide phosphate solution given by intravitreal route (Table 4-1). The laser-aiming beam was focused on the Bruch's membrane. The aim was to rupture the Bruch's membrane so the choroidal blood vessel would invade the subretinal space forming choroidal neovascularization. The sure sign of the Bruch's membrane rupture by laser was the formation of bubbling at the site of the laser application with or without hemorrhage. An intravitreal administration was applied 2 days after photocoagulation.

Treatment Administration

Intravitreal injection of triamcinolone nanoparticles (1 μl) containing 800 μg/ml drug was performed in the right eye. The mice were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (40 mg/kg). Different treatments were injected into the vitreous under a

dissecting microscope. A 27-gauge needle was first used to make an incision 0.5 mm posterior to the temporal limbus and the Hamilton needle was inserted through the incision, approximately 1.5 mm deep, and angled toward the optic nerve until the tip of needle was seen in the center of the vitreous. Intraperitoneal injection treatment group a dose of approximately 160 μg of drug was given.

Preparation of Flat Mounts

Two weeks after laser treatment, the size of choroidal neovascularization lesions were evaluated. Mice used for the flat-mount technique were anesthetized and perfused with 3 ml of phosphate-buffered saline containing 50 mg/ml of fluorescein-labeled dextran. The eyes were removed and fixed for 2 hour in 10% phosphate-buffered formalin. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup. Radial cuts (4 to 7, average 5) were made from the edge of the eyecup to the equator and the eyecup was flat-mounted in aquamount with the sclera facing down and the choroid facing up. Figure 4-1 shows the flat mounts of the choroid. Flat mounts were examined by fluorescence microscopy. All images of neovascularization sites were obtained at 40X objective with a Carl Zeiss Axioplan2 imaging channel. The neovascularization was quantified by image J software.

ImageJ is a public domain, Java-based image processing program developed at the National Institutes of Health. ImageJ can display, edit, analyze, process images. Image J calculates area and pixel value statistics of user-defined selections.

Statistical Analysis

One way ANOVA analysis followed by Dunnette post test was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) with p value less than 0.05 considered as significant.

Results

Figure 4-3 shows the neovascularization area measured for various treatments. The control group showed mean choroidal neovascularization area of 6002 ± 2693 and 3025 ± 2085 μm^2 in the left and the right eye respectively. All the treatments were compared with the control group left eye. No statistically significant difference was seen with intravitreal delivery of triamcinolone acetonide phosphate solution. Intravitreal injection of triamcinolone acetonide nanoparticles had developed only 10% (614±71 μm^2) neovascularization in the treated eye. The contralateral eye also showed reduced neovascularization of only 21% (1296±57 μm^2) compared to the control. A significantly reduced choroidal neovascularization was also seen with intraperitoneal administration of triamcinolone acetonide nanoparticles showing a mean area of neovascularization of 580 ± 303 and 943 ± 601 μm^2 in the left and the right eyes respectively. Figure 4-2 shows laser induced choroidal neovascularization images of the various treatments.

Discussion

The specific stimuli for choroidal neovascularization are not fully understood. It generally occurs in the presence of a pro-inflammatory and pro-angiogenic environment. It is characterized by elevated levels of angiogenic factors. The appearance of choroidal neovascularization can cause vision loss and the current treatments have limited effectiveness. Recently there is great interest in identifying clinically relevant inhibitors of ocular neovascularization and in the development of effective treatments targeting choroidal neovascularization.

As the pathology of choroidal neovascularization is better understood, therapeutic agents that can treat the cause of choroidal neovascularization are being considered. Growth factors and cell adhesion molecules that have been implicated in neovascularization include: intercellular adhesion molecular (ICAM-1), basic and acidic fibroblast growth factor (aFGF and bFGF), [118]

and vascular endothelial growth factor (VEGF) [119]. Previous studies have demonstrated that VEGF is an angiogenic factor [36] that is upregulated in and localized to the CNV lesion suggesting that VEGF plays an important role in choroidal neovascularization formation. Previous experiments have shown that sustained intravitreal delivery of VEGF in animal models can cause widespread retinal vascular dilation and compounds with anti-VEGF properties effectively suppress CNV.

Although the principal effects of corticosteroids are the stabilization of the blood-retinal barrier, resorption of exudation and downregulation of inflammatory stimuli, they are also potent inhibitors of neovascularization [120]. A number of studies have shown that corticosteroids inhibited preretinal and subretinal neovascularization; however, its mechanisms of antiangiogenesis remain unknown. Several researchers have suggested that corticosteroids can act on the neovascular cascade by processes such as directly decreasing levels of VEGF, inhibiting bFGF-induced migration and tube formation in choroidal microvascular endothelial cells, inhibiting extracellular matrix turnover by downregulating metalloproteinase-2 (MMP-2) production, downregulating intercellular adhesion molecule-1 (ICAM-1) expression, and reducing major histocompatibility complex- II antigen expression [38].

In this study, we evaluated the effect of corticosteroid nanoparticles on laser induced choroidal neovascularization in mice model. Neovascularization was stimulated in mice using high energy laser. High energy laser causes a rupture of the Bruch's membrane; thereafter, under the influence of various angiogenic factors, an ingrowth of choroidal vessels under the retinal pigment epithelium and then into the subretinal space develops. Although, in this model, pathogenesis of the neovascularization is different from age-related macular degeneration, the formation of CNV is believed to follow the similar pattern and identical angiogenic factors such

as VEGF [121] and bFGF [122] are expressed by the retinal pigment epithelium and endothelial cells.

Our observations showed that the formation of vascular tubes in CNV was blocked by triamcinolone acetonide nanoparticles *in vivo* in mice (Figure 4-3). Considering the reduced size of CNV in the treated group compared with the control group, it is likely that the proliferation and/or migration of vascular endothelial cells are inhibited in corticosteroid treated eyes. Given that VEGF production was found to be suppressed by triamcinolone acetonide treatment in our cell culture studies and by others *in vivo* [123], it is likely that angiogenesis inhibitory action of triamcinolone is due to decrease of VEGF directly or indirectly. The effect of Kenalog (triamcinolone acetonide injectable suspension) which is currently used by ophthalmologists was studied by others in our group and only showed 15% neovascularization compared to the control group (unpublished). This indicates that triamcinolone acetonide retained its therapeutic effect when formulated as nanoparticles

A significant decrease was seen in both the treated eye and the contralateral eye but the choroidal neovascularization is comparatively less in the treated eye suggesting some targeted delivery of nanoparticles was achieved.

Also choroidal neovascularization was decreased with intraperitoneal route of administration indicating the effect on the untreated eye might be due to the systemic entry of the drug from the treated eye. The targeted delivery may be further improved by further decreasing the rate of drug release and determining the therapeutic concentration required to treat neovascularization. So far there is no information regarding the therapeutic range of concentration of intraocular triamcinolone for antiproliferative effect.

Our studies showed reduced choroidal neovascularization with triamcinolone acetonide nanoparticle formulation and with Kenalog, both of which release the drug slowly but no significant decrease was seen with triamcinolone acetonide phosphate solution. This implies that sustained release is essential for inhibition of choroidal neovascularization.

In this study, we demonstrated that intravitreal triamcinolone acetonide poly(lactide-co-glycolide) nanoparticles can inhibit the development of experimental choroidal neovascularization. It further supports previous animal studies that have indicated that corticosteroids can inhibit choroidal neovascularization formation [124].

Conclusions

In conclusion, our results indicate a notable inhibitory effect of triamcinolone loaded nanoparticles on laser-induced choroidal neovascularization in the mice model. However, the efficacy of this drug for the treatment of human CNV requires further investigation. We believe that triamcinolone acetonide loaded polymer nanoparticles could be promising to inhibit human neovascularization. The use of corticosteroid nanoparticles will help to deliver effective dose to the posterior parts of the eye and prevent repeated intravitreal injection as they can sustain the drug release and can also ensure better visibility resulting in patient compliance. It should also be noted that this study, by its design, demonstrated inhibition or prevention of choroidal neovascularization, but not regression of pre-existing neovascularization, which would be more relevant to humans.

Table 4-1. Treatment groups

Treatment	Route
Triamcinolone acetonide nanoparticles	Intravitreal
Triamcinolone acetonide nanoparticles	Intraperitoneal
Triamcinolone acetonide solution	Intravitreal
Blank nanoparticles	Intravitreal

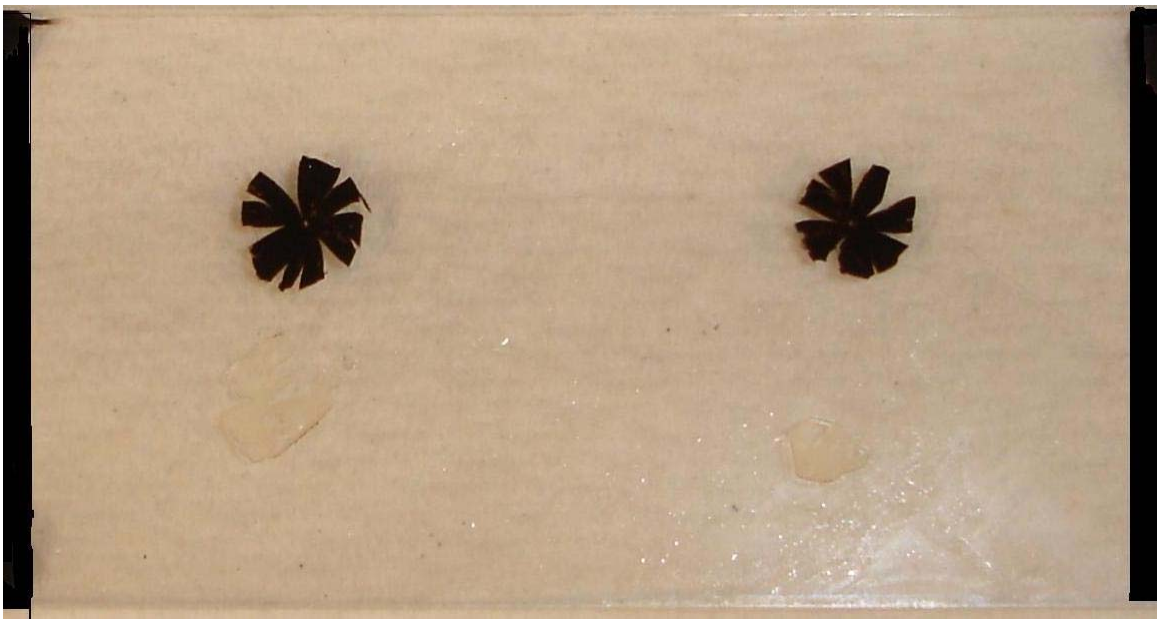


Figure 4-1. Choroidal flat mounts of the left and right eye

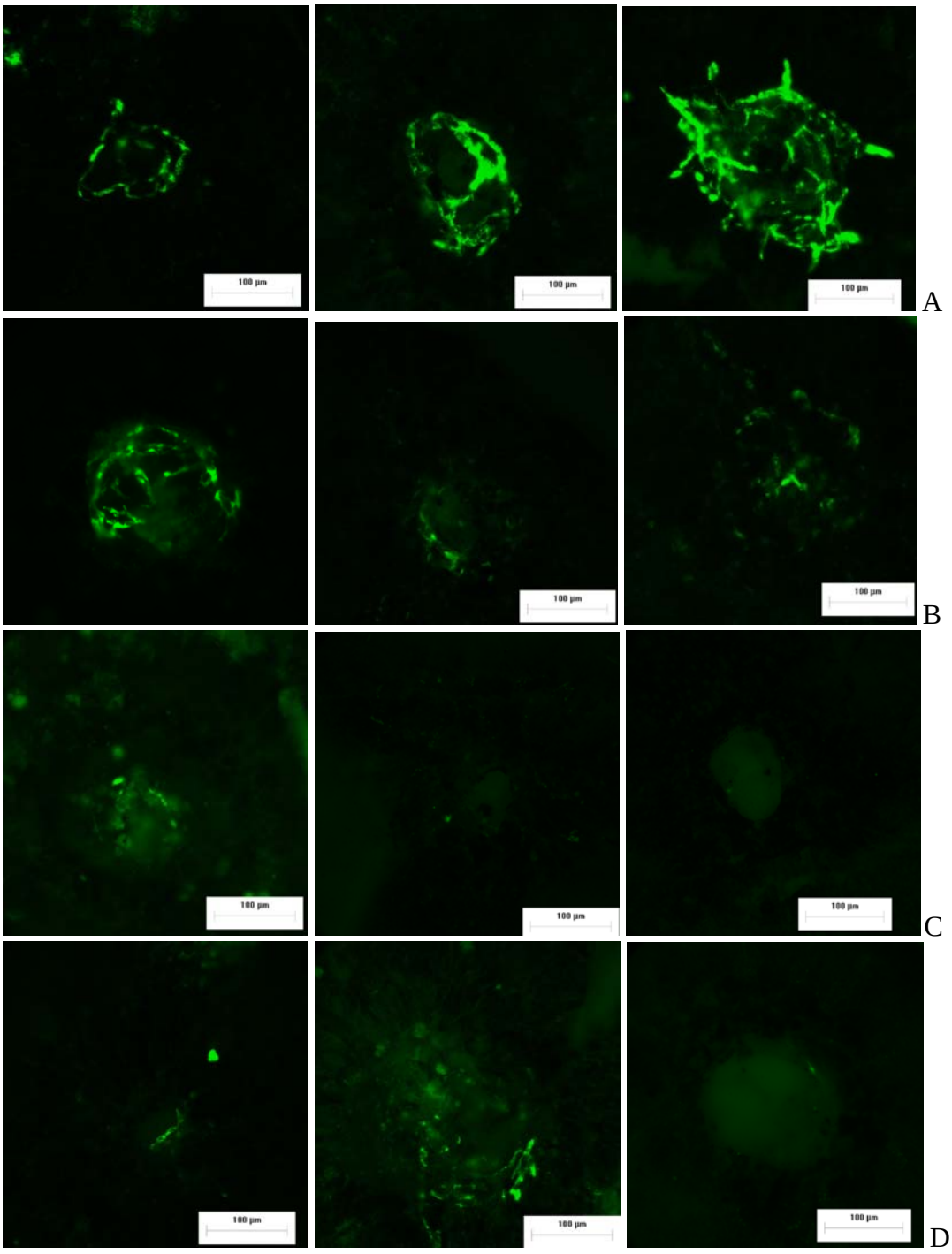


Figure 4-2. Representative images of laser induced choroidal neovascularization (A) control group (B) Intravitreal injection triamcinolone solution (C) Intraperitoneal injection of triamcinolone nanoparticles (D) Intravitreal administration of triamcinolone nanoparticles

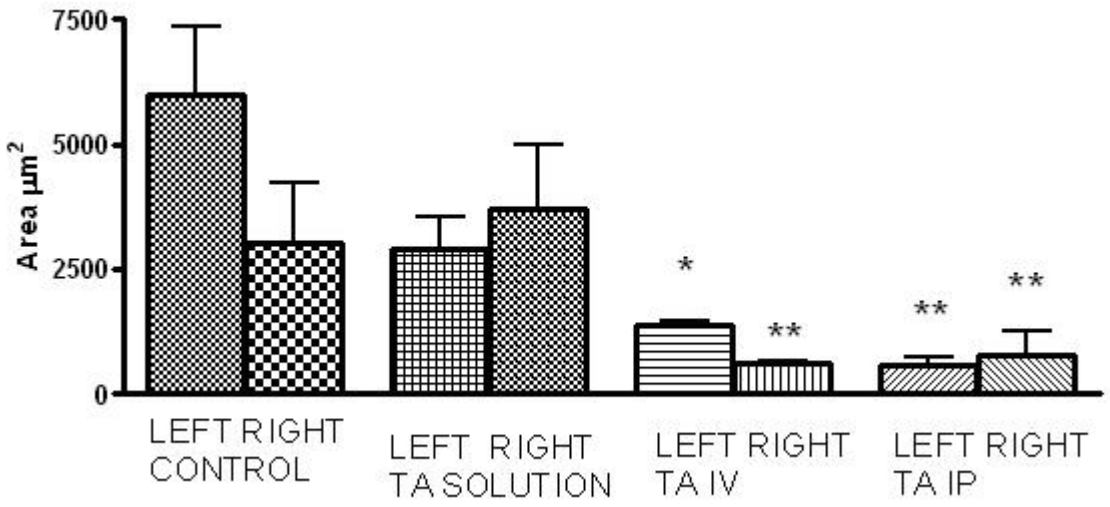


Figure 4-3. Neovascularization area of four treatment groups. P< 0.05 was considered statistically significant *P < 0.05, **P < 0.01

CHAPTER 5
LOTEPREDNOL ETABONATE NANOPARTICLES AND THEIR EFFECT ON LASER INDUCED CHOROIDAL NEOVASCULARIZATION IN MICE

Introduction

Corticosteroids are the mainstay of therapy for inflammatory conditions. Traditionally used corticosteroids are associated with two clinically important ocular side effects: cataract and increased intraocular pressure [43]. Hence, there is a need for therapeutic agent with efficacy comparable to that of currently available corticosteroids, but without the adverse effects. The continuing development of ophthalmic steroids has resulted in compounds that have a low tendency to raise intraocular pressure (IOP).

Loteprednol etabonate is a corticosteroid which is the product of soft drug design (a compound that undergoes predictable metabolism to inactive metabolites) [125]. It has been developed as a topical treatment for ocular inflammation. Loteprednol etabonate is designed to be rapidly converted to inactive and nontoxic metabolites, thus minimizing systemic adverse effects. Loteprednol has been approved by the Food and Drug Administration (FDA) as the active ingredient of three ophthalmic preparations (Lotemax, Alrex, Zylet) [125-127]. At present, it is the only corticosteroid approved by the FDA for use in all inflammatory and allergy-related ophthalmic disorders.

The effect of loteprednol etabonate on neovascularization has not been studied. In this study, we investigate the effect of loteprednol on choroidal neovascularization in the posterior region of the eye. Loteprednol is very lipophilic in nature and if given in the present form will be cleared from the vitreous fast and may not show any therapeutic effect. Nanoparticle formulation of the drug will sustain the drug release and hence can be used to treat conditions in the posterior region of the eye without the adverse effects associated with other steroids.

The goals of this study are:

- determine the effect of intravitreal delivered loteprednol etabonate on intraocular pressure in rabbits
- determine the effect of loteprednol on VEGF secretion in ARPE-19 cells
- prepare and characterize loteprednol etabonate nanoparticles
- determine the effect of loteprednol and its nanoparticles on ARPE-19 cell viability
- finally to investigate the effect of intravitreal delivered loteprednol nanoparticles on laser induced choroidal neovascularization

Materials and Methods

Intraocular Pressure Measurements in Rabbits

Initially 2-3 drops of topical anesthetic was applied to the eye and the initial intraocular pressure (IOP) was measured with a pneumatonometer, this will represent the preinjection IOP. Later the animals were anesthetized with intramuscular injection of 35 mg/Kg of Ketamine and 5 mg/Kg of Xylazine. Steroid (100 μl) was injected using tuberculin syringe with a 27-gauge needle into the vitreous cavity of the right eye. The needle was pointed toward the optic nerve to ensure that the lens was not inadvertently nicked. As a control, the vehicle-only solution was injected in the other eyes at the same time. A cotton swab was placed immediately as the needle was withdrawn, to insure no reflux of steroid occurred. The eye was vigorously massaged to reduce the intraocular pressure because of increased volume resulting from the injection. This was done to reduce the IOP to a level within a few points of pre-injection The IOP was then measured on 1, 5, 10, 15, 20 and 30 days.

Preparation and Characterization of Loteprednol Etabonate Nanoparticles

Materials and methods are similar to that described in chapter 2 for characterization of nanoparticles based on size, encapsulation efficiency and *in vitro* release. Briefly, the nanoparticles were prepared by solvent evaporation method and the prepared nanoparticles were characterized for size, shape, encapsulation and *in vitro* drug release. *In vitro* release studies

were performed with 500 μg of the drug in the dialysis bag, sink condition was maintained with 1% sodium dodecyl sulfate. The drug content in encapsulation studies was measured using HPLC. Acetonitrile and water (50:50) mobile phase was used with flow rate of 1 ml/min. A C-18 column was used and UV detection was accomplished at 254 nm. Stock solution of 100 μg/ml was prepared in acetonitrile. To determine the encapsulation efficiency the calibration curve was obtained from standards 1 to 50 μg/ml ($r^2 > 0.99$).

In vitro drug release studies, acetonitrile: water: acetic acid (50:49.5:0.5 vol/vol) was used as the mobile phase. Calibration curve was obtained from standards 1 to 30 μg/ml in the release media ($r^2 > 0.99$).

Effect of Loteprednol Etabonate on VEGF Secretion in ARPE-19 Cells

Materials and method used are similar to that described in chapter 3 for effect on VEGF in ARPE-19. Briefly, ARPE-19 cells were seeded onto a 24 well plate and allowed to grow to confluence. The monolayers are incubated with loteprednol 10 μM. The culture media is collected at the end of 12 hours. The secreted VEGF in supernatants is quantified by ELISA method capable of detecting VEGF 165 and the VEGF secretion was normalized to total protein assayed using BCA kit after lysing the cells.

Cell Toxicity of Loteprednol Etabonate and its Nanoparticles on ARPE-19 Cells

Materials and method used are similar to that described in chapter 3 for cell toxicity study ARPE-19 cells were seeded at a density of 10,000 cells/well and were allowed to attach overnight in 96-well plates. The cells were then exposed to loteprednol or its nanoparticles (1, 10 and 100 μM). At the end of 24 hour incubation, the cell viability was determined by MTT assay. The cell viability is then determined by the relative formazan formation after corticosteroid and the nanoparticles treatments compared to the control group.

Effect of Loteprednol Etabonate Nanoparticles on Laser Induced Choroidal Neovascularization in mice

Materials and methods are similar to that described in chapter 4. The mice were anaesthetized and the pupils were dilated. The mice were lasered with 532-nm diode laser photocoagulation with 300 mW power for 200 msec duration, with a mean diameter of 50 μm, with three lasers to each eye. Treatment groups included (n=5) blank nanoparticles, loteprednol nanoparticles and triamcinolone nanoparticles delivered through intravitreal route in the right eye. After 2 weeks the mice were sacrificed and flat mounts were prepared and the area of choroidal neovascularization was determined using image J. Image J is a public domain, Java-based image processing program developed at the national institutes of health.

Statistical Analysis

All statistical analyses, T-test and ANOVA were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) with p value less than 0.05 considered as significant.

Results

Intraocular Pressure Measurements in Rabbits

Mean intraocular pressure (IOP) data of triamcinolone acetonide and loteprednol etabonate (not nanoparticles) are presented in figure 5-1. Treatment-related differences in IOP were not apparent in our study. No other statistically significant differences were seen during the study. Mean IOP did not appear to be significantly affected by intravitreal administration of triamcinolone or loteprednol.

Preparation and Characterization of Loteprednol Nanoparticles

The nanoparticles prepared were spherical and had a smooth surface. The size of the particles was 196 ± 32 nm with good encapsulation efficiency of 72.56 ± 1.75% (table 5-1). *In vitro* release studies showed sustained drug release with the nanoparticles releasing the drug at

slower rate compared to loteprednol etabonate alone (figure 5-2). Extrapolated results showed 100% drug release occurs in 25 days (figure 5-3).

Effect of Loteprednol on VEGF Secretion in ARPE-19 Cells

Figure 5-4 shows the effect of loteprednol on VEGF secretion. Loteprednol showed statistically significant decrease of VEGF secretion at 10 μM concentration. Compared to no treatment, the VEGF secretion was reduced by 52%.

Cell Toxicity of Loteprednol and its Nanoparticles on ARPE-19 Cells

The effect of loteprednol etabonate and its nanoparticles was studied on cell viability of ARPE-19 cells. Figure 5-5 shows that loteprednol and its nanoparticles did not show any significant decrease in cell viability across the entire range from 1 to 100 μM at the end of 24 hours.

Effect of Loteprednol Nanoparticles on Laser Induced Choroidal Neovascularization in Mice

Loteprednol nanoparticles were able to inhibit choroidal neovascularization. Compared to control group that showed a mean area of $2615 \pm 1954\ \mu m^2$ and $2151 \pm 1228\ \mu m^2$ in the left and right eye respectively, loteprednol nanoparticles showed only 39% ($1006 \pm 833\ \mu m^2$) and 28% ($731 \pm 466\ \mu m^2$) choroidal neovascularization in the left and the right eye, respectively. Triamcinolone nanoparticles showed choroidal neovascularization of 379 ± 217 and $180 \pm 140\ \mu m^2$ choroidal neovascularization compared to the control.

Discussion

Corticosteroids like triamcinolone acetonide can treat neovascularization but are associated with adverse effects. These adverse affects include elevations in intraocular pressure and the formation of, or acceleration of the development of, cataracts [42]. Elevations in intraocular pressure are of particular concern in patients who are already suffering from elevated intraocular

pressure, such as glaucoma patients. Moreover, a risk exists that the use of corticosteroids in patients with normal intraocular pressure will cause elevation in pressure that result in damage to ocular tissue. Since therapy with corticosteroids is frequently long term, i.e., several days or more, a potential exists for significant damage to ocular tissue as a result of prolonged elevations in intraocular pressure attributable to that therapy.

Increased IOP is encountered with about 30% of patients using steroids [128], and is reported to develop about 2 months after administration of triamcinolone [129]. Cataract is reported in about up to 38% of patients receiving systemic corticosteroids [129], usually manifesting after 2 months to 1 year of exposure. The absence of significant effects of intravitreal injection of triamcinolone on intraocular pressure in rabbits seen in our study might be due to the delayed onset of intraocular pressure. Loteprednol etabonate has a lower propensity to induce elevation in intraocular pressure even when used in known steroid responders [130].

The pathophysiology behind steroids and raised IOP is at the level of the trabecular meshwork whereby mucopolysaccharides from free-floating steroids bind to the ultrastructure of the trabecular meshwork and reduce the pore size and thus, reduce aqueous outflow. Steroids that typically increase intraocular pressure are the ones that have a ketone group in position 20 of the steroid skeleton. The ketone on the C 20 of steroids is also believed to be responsible for the formation of Schiff bases between the steroid C 20 with nucleophilic groups such as -amino groups of lysine residues of proteins [131] resulting in cataract.

Loteprednol etabonate appears to have an improved safety profile compared to ketone corticosteroids, and may be more suitable than ketone corticosteroids for the treatment of conditions in which long-term therapy is necessary [132]. It is not completely known why loteprednol causes less intraocular [133] pressure increase than other corticosteroids. One of

reasons is that the predictable intraocular conversion of the drug to an inactive compound may reduce the amount of active corticosteroid in the trabecular meshwork.

Nanoparticle formulation of loteprednol helps in sustaining the drug release and prevents the rapid elimination of the drug. The nanoparticles had slower release compared to triamcinolone nanoparticles prepared with similar parameters. The difference in the release period of these two drugs might be due to the lipid solubility of the drugs. Lipophilic drugs can distribute more homogeneously in the matrix of polymer poly(lactide-co-glycolide), and can be released for a longer time.

Our studies also showed that loteprednol has anti-angiogenic effect as it was able to inhibit secretion of VEGF, a growth factor that stimulated new blood vessel growth and hence can be used to treat neovascular conditions. Loteprednol nanoparticles reduced the occurrence of choroidal neovascularization in mice model. But the decrease was less compared with triamcinolone acetonide. The rate of drug release from the loteprednol nanoparticles might need to be optimized for higher release rate to show a better therapeutic effect. The decreased therapeutic effect might also be due to loteprednol being metabolized within the nanoparticles by the various esterases in the eye.

Conclusions

Loteprednol etabonate inhibited VEGF secretion responsible for neovascularization in human retinal pigment epithelial cells. Nanoparticles formulated had better encapsulation and slower release than triamcinolone acetonide nanoparticles. Loteprednol or its nanoparticles did not cause toxicity in retinal pigment epithelial cells and hence might be safer compared to triamcinolone acetonide for chronic use. Loteprednol nanoparticles were able to reduce laser induced choroidal neovascularization in mice. Although the formulation needs to be further

optimized loteprednol nanoparticles might be a good option to treat neovascular conditions in the posterior eye.

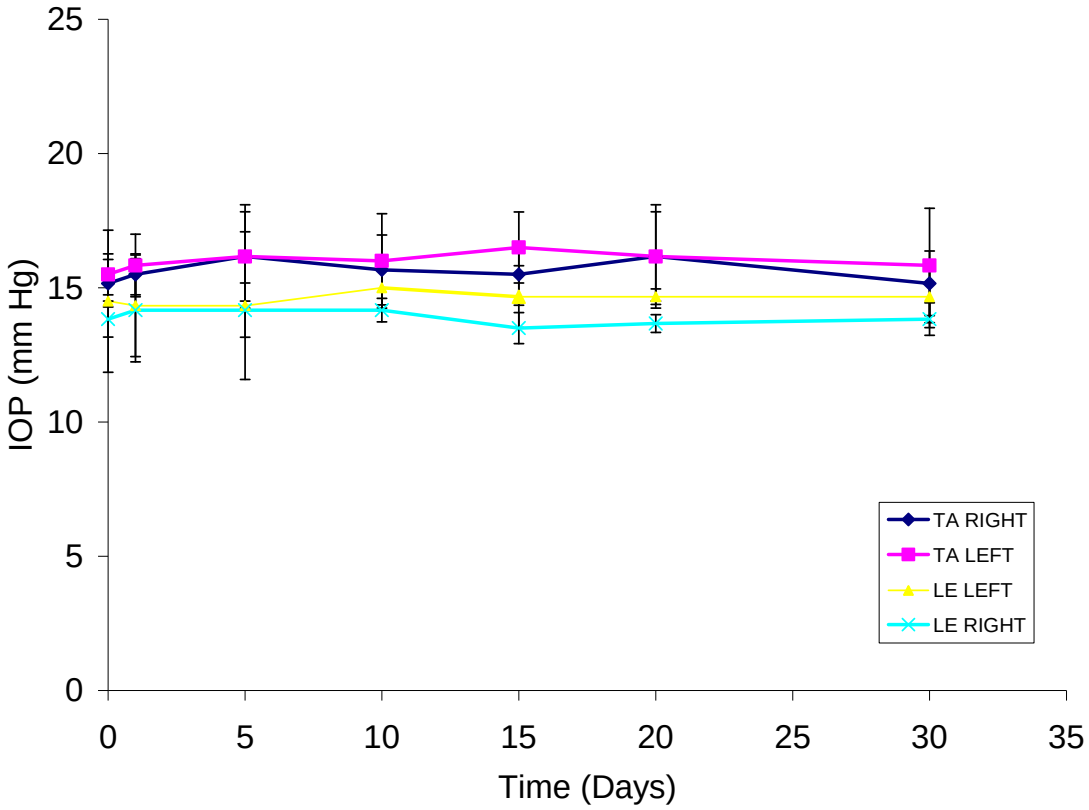


Figure 5-1. Intraocular pressure measurements (IOP) for a period of 30 days in rabbits after intravitreal injection of triamcinolone (TA) and loteprednol (LE). No statistically significant difference was seen.

Table 5-1. Size and encapsulation efficiency of loteprednol nanoparticles

Formulation	PVA	Drug:Polymer	Size (nm)	Drug Loading (%wt/wt)	EE%
LE NP	1%	1:10	196 ± 32	10.09 ± 2.2	72.56 ± 1.75

Table 5-2. Zero order and Higuchi equations of *in vitro* drug release showing the effect of polymer

Formulation	Zero Order		Higuchi Equation	
LE	R^2 = 0.9709	Y= 0.3291X+ 1.2076	R^2 = 0.9722	Y= 5.9567X+26.263
LE NP	R^2 = 0.9826	Y= 0.2148X +4.9107.	R^2 = 0.9922	Y= 4.2963X + 24.769

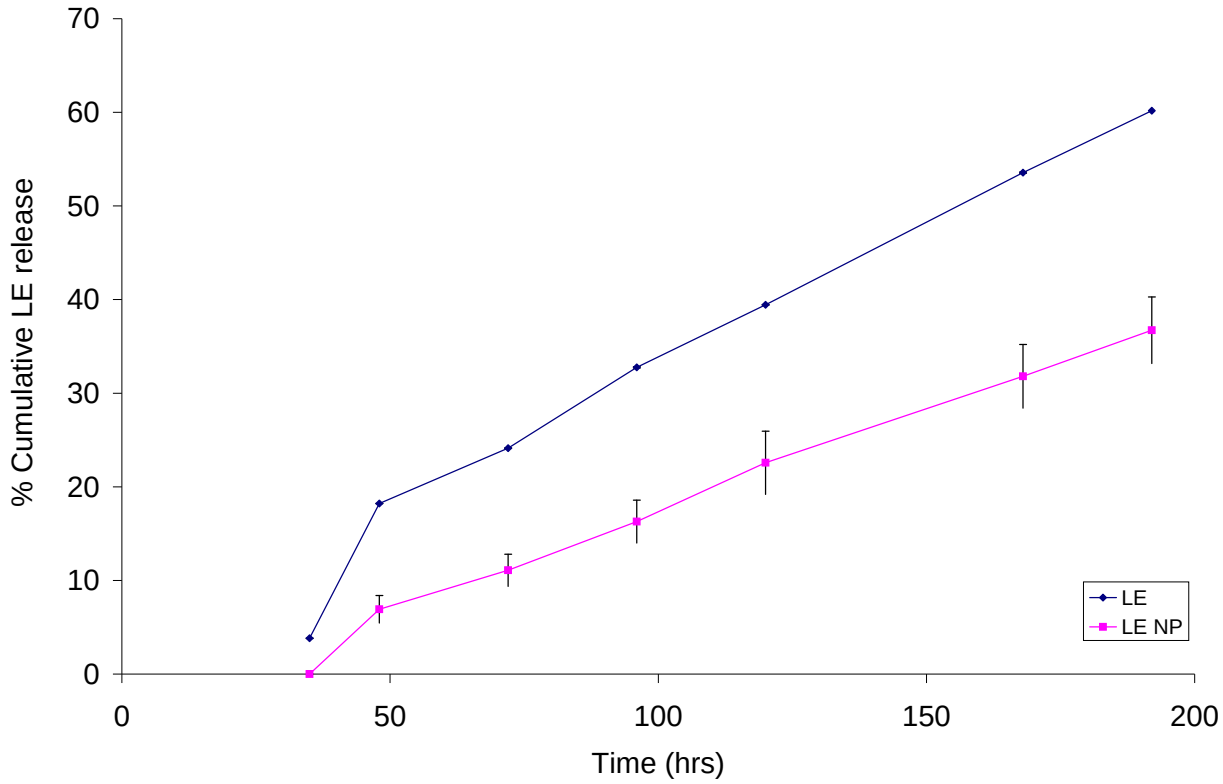


Figure 5-2. *In vitro* release data of loteprednol etabonate and its nanoparticles. All the studies were performed at 37°C. Data are expressed as the mean ± SD of results in two experiments

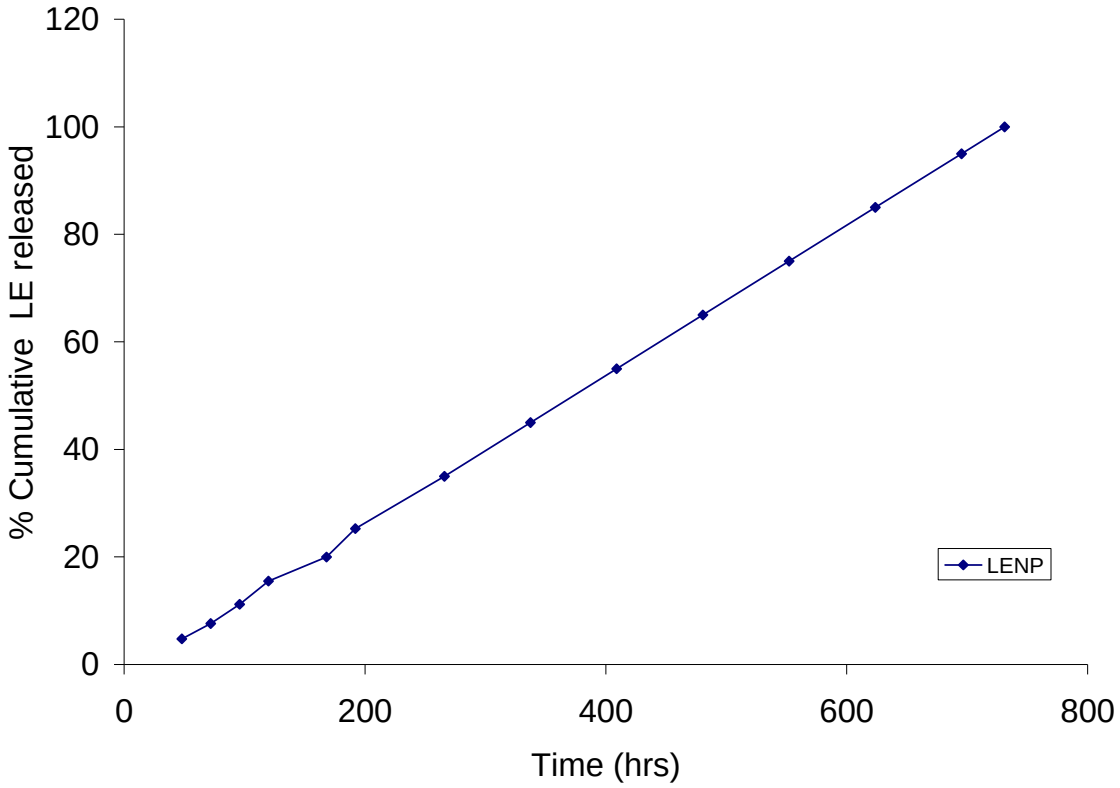


Figure 5-3. *In vitro* release studies of loteprednol nanoparticles extrapolated to 100% cumulative drug release

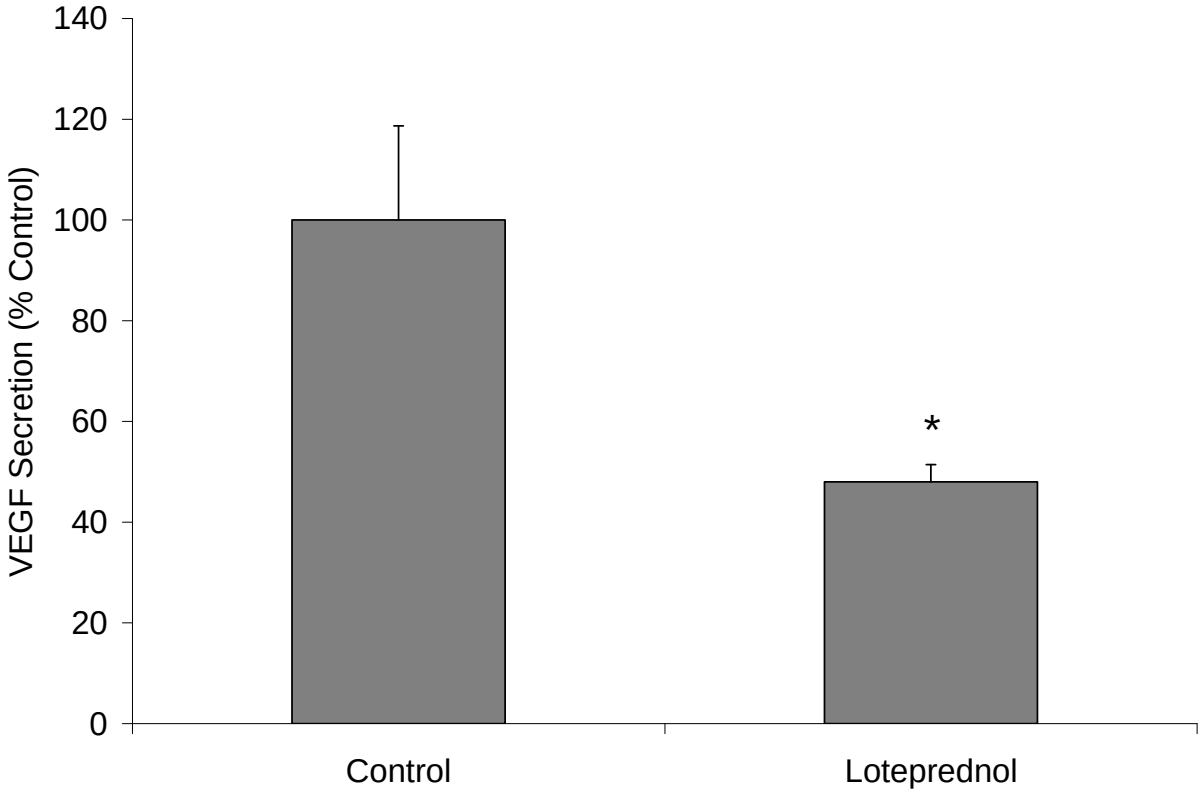


Figure 5-4. Loteprednol etabonate inhibited secretion of VEGF in ARPE-19 cells at concentration 10 μM (N=8).Data are expressed as the mean ± SD. * Significantly different from the control at $P < 0.05$

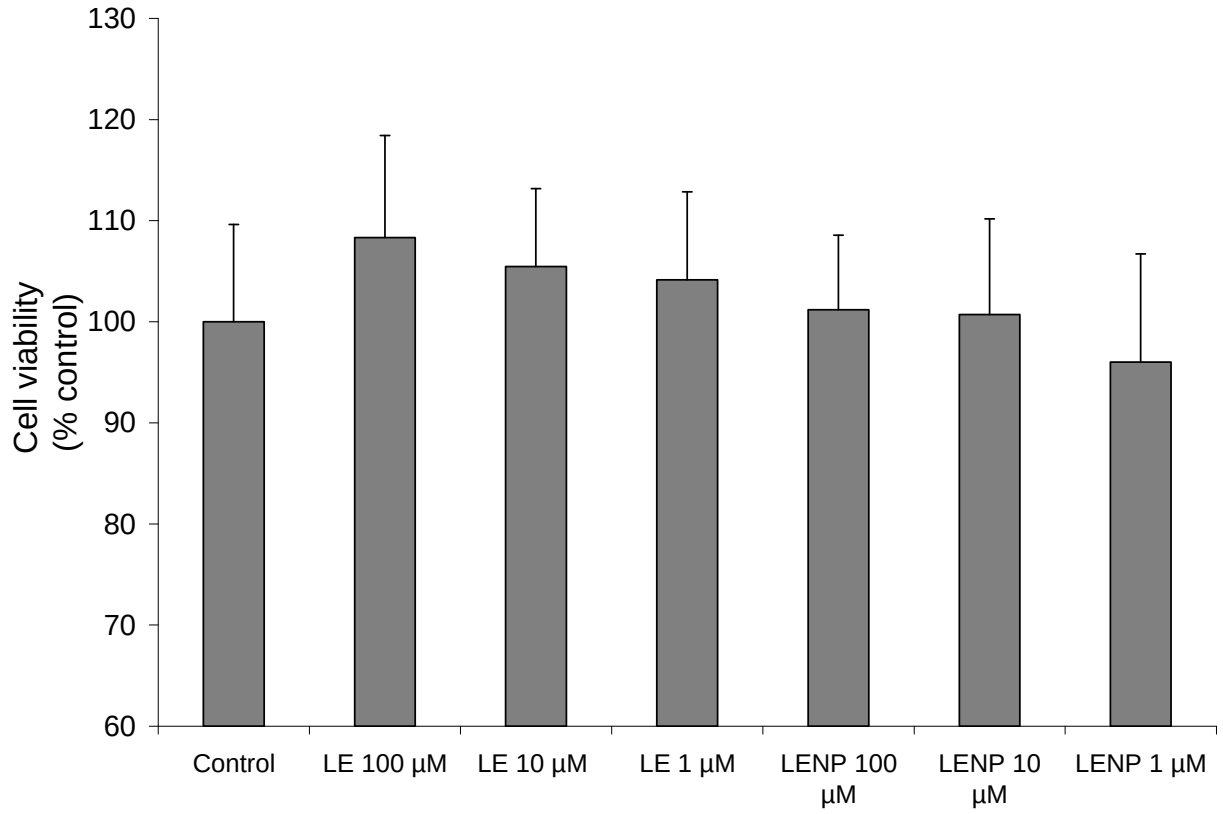


Figure 5-5. Effect of loteprednol (LE) and loteprednol nanoparticles (LE NP) on viability of ARPE-19 Cells after 24 hours. Treatments as determined by MTT assay. Data as mean ± SD (N=8). * Significantly different from the control at $P < 0.05$.

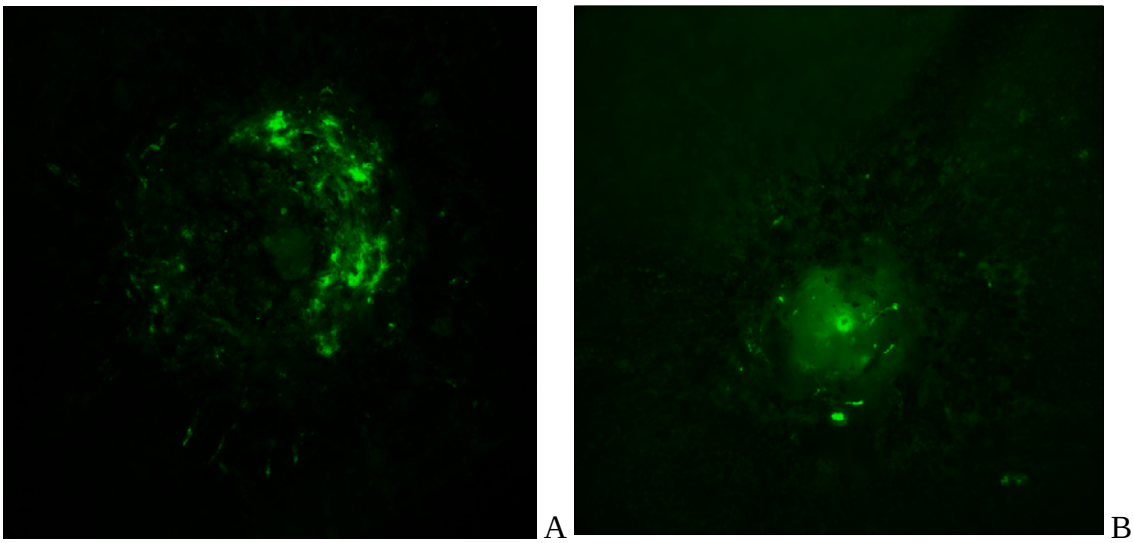


Figure 5-6. Representative images of laser induced choroidal neovascularization (A) control group (B) loteprednol nanoparticles treated groups

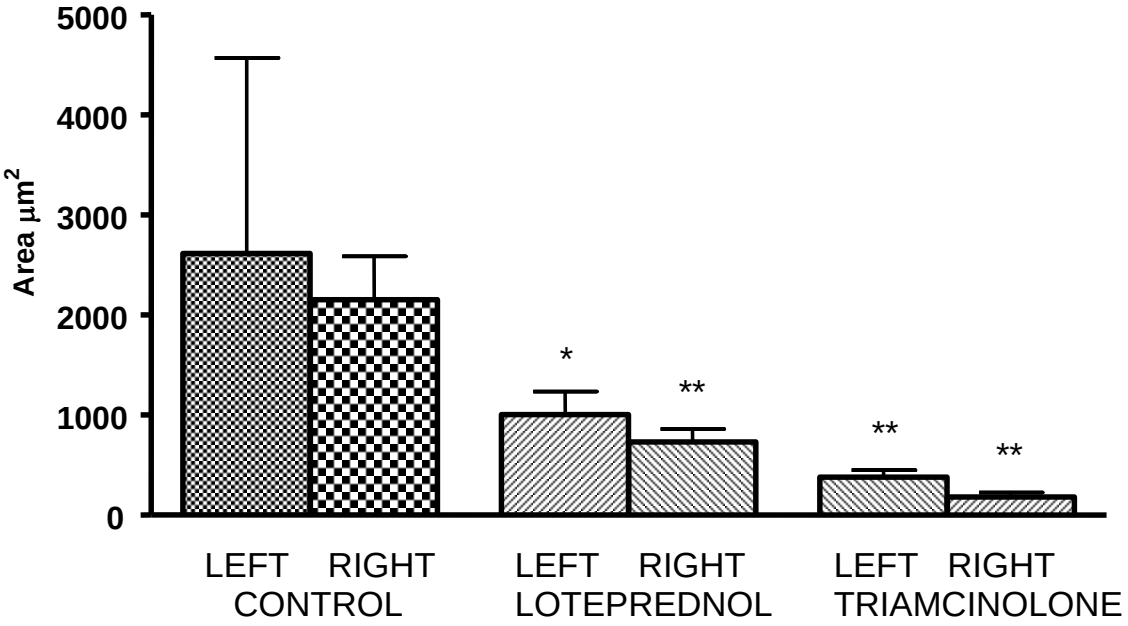


Figure 5-7. Neovascularization area of blank nanoparticles, loteprednol nanoparticles, triamcinolone nanoparticles in the left (untreated) and right (treated) eyes. Data as mean ± SD (N=5). * Significantly different from the control at $P < 0.05$. *$P < 0.05$, **$P < 0.01$.

CHAPTER 6
CONCLUSIONS AND FUTURE WORK

In this dissertation we addressed the use of nanoparticles of corticosteroids to treat conditions occurring in the posterior region of the eye, which are a major cause of blindness among the elderly. Drug delivery to treat diseases like age-related macular degeneration and diabetic retinopathy is challenging. It is difficult to attain therapeutic concentration to treat them due to long diffusional distance and presence of ocular barriers. Intravitreal delivery offers the most direct approach to deliver nanoparticles to the target area.

Corticosteroid nanoparticles below 500 nm were successfully prepared using emulsion solvent evaporation technique. The nanoparticles of triamcinolone and loteprednol had good encapsulation efficiency and had longer drug release compared to the micronized drugs alone. Nanoparticles prepared were able to sustain the drug release as was shown in chapter 2 and hence can prevent repeated injections.

The use of nanoparticle drug delivery system for triamcinolone acetonide showed a decrease in toxicity in ARPE-19 cells compared to the drug itself. Neither loteprednol nor its nanoparticles caused cell toxicity and hence are better suited for prolonged therapy. The cellular uptake studies showed that the nanoparticle uptake was time and dose dependent. Both triamcinolone acetonide and loteprednol were able to inhibit VEGF secretion responsible for neovascularization.

Triamcinolone acetonide maintained its therapeutic effect in the nanoparticle formulation inhibiting laser induced choroidal neovascularization in mice. Although some targeted delivery was achieved in the treated eye the formulation has to be further optimized based on the therapeutic concentration required. Determining the systemic concentration of the drug and the

understanding the transport of the drug to the contralateral eye will help to achieve better targeted delivery.

In our study loteprednol nanoparticles showed less effect on choroidal neovascularization compared to triamcinolone nanoparticles. To show that the decreased effect is due slower release pharmacokinetic studies need to be conducted to determine the concentration of the drug in various tissues and the efficacy has to be shown over long term with no toxicity.

In this study we showed that nontoxic corticosteroid nanoparticles pretreatment was able to prevent laser induced choroidal neovascularization. The use of corticosteroid nanoparticles were able to deliver effective dose to the posterior region of the eye and help prevent repeated intravitreal injection. Future studies need to address the effect of corticosteroid nanoparticles on existing neovascular conditions.

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BIOGRAPHICAL SKETCH

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