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TITLE: Development of Nanopharmaceutical Therapy for Combat-Related Proliferative Vitreoretinopathy

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14. ABSTRACT

Objective: The objective of this project is to develop anti-fibrotic therapy using pirfenidone-loaded nanoparticles to treat proliferative vitreoretinopathy (PVR), a specific type of intraocular fibrosis, in a rabbit model of penetrating eye injury.

Background

Intraocular fibrosis after penetrating eye injury is a leading cause of vision loss among members of the armed forces, whom are vulnerable to penetrating eye injuries due to exposure to high velocity projectiles from detonations of IEDs. Current management of PVR and intraocular fibrosis involves the surgical removal of the intraocular fibrotic membrane. However, despite the 90% anatomic surgical success rate, membranes frequently regrow causing retinal detachment and vision loss, which makes PVR difficult to treat. Pirfenidone is a promising pharmaceutical agent approved for the treatment of other types of fibrosis, including pulmonary fibrosis and renal fibrosis. Although pirfenidone has been shown recently as a possible treatment for intraocular fibrosis, there are concerns associated with its dosage and number of injections. Thus, to develop pirfenidone as a clinical therapy for PVR, the delivery of the drug must be engineered as to improve its bioavailability and delivery to the posterior segment of the eye. Success of this project will be a significant advance for not only treatment of PVR, but also for the treatment of other disorders that are localized to the posterior ocular segment.

Hypothesis

The hypothesis of this proposal is that intravitreally injectable Avidin-Fatty Acid (AFA) PLGA nanoparticles, engineered with chemical and physical properties to target specific ocular cells, will provide an effective platform for delivery of pirfenidone to the posterior ocular segment leading to the prevention of intraocular fibrosis in rabbits following penetrating eye injury.

Specific Aims

Specific Aim 1. Development, characterization, and optimization of targetable AFA-PLGA nanoparticles for sustained delivery of pirfenidone to the posterior segment of the eye.

Specific Aim 2. Evaluation of pirfenidone-loaded AFA-PLGA nanoparticles *in vitro* for determination of biocompatibility and pharmacodynamics of release.

Specific Aim 3. Assessment of *in vivo* safety, pharmacokinetics (PK), and bioefficacy of intravitreally injected pirfenidone-loaded AFA-PLGA nanoparticles.

Study Design

Aim 1: Pirfenidone will be loaded into AFA-PLGA nanoparticles, which will be evaluated for chemical and physical properties as well as release kinetics.

Aim 2: Retinal pigment epithelial cells will be treated with pirfenidone-loaded AFA-PLGA nanoparticles to evaluate anti-fibrotic functions and cytotoxicity.

Aim 3: Rabbits with penetrating eye injury will receive intravitreal injections of pirfenidone-loaded AFA PLGA nanoparticles. The nanoparticles will be tracked to ensure delivery to the posterior segment. The rabbit eyes will be scored for severity of intraocular fibrosis to compare treated versus untreated eyes.

15. SUBJECT TERMS NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	14	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

1. INTRODUCTION

Posterior penetrating eye injury is a common battlefield-related ocular injury. Improper retinal wound healing can result in intraocular fibrosis which leads to severe visual impairment. Although there have been numerous technical advances in surgical management to repair the retina, the continuous scarring in the eye after surgical intervention eventually leads to total vision loss or blindness. This suggests that pharmacological treatment is needed. Pirfenidone is a promising anti-scarring agent that has been approved for the treatment of pulmonary fibrosis and has also shown great potential in the prevention of liver or renal fibrosis. The objective of this study is to develop novel biodegradable nanoparticles for the sustained delivery of pirfenidone to the posterior segment of the eye. The therapeutic effects of pirfenidone-loaded nanoparticles for the prevention and treatment of retinal scarring will be verified using a rabbit model of posterior penetrating eye injury.

2. KEYWORDS

Proliferative Vitreoretinopathy (PVR), Intraocular Fibrosis, Pirfenidone, poly lactic-co-glycolic acid (PLGA) Nanoparticles, Posterior Penetrating Eye Injury

3. ACCOMPLISHMENTS

What were the major goals of the project? (Goals to be accomplished and status.)

The long-term goal of this project is to develop novel nanoparticles for the delivery of therapeutics to treat PVR. The project objectives are 1) to develop functionalized biodegradable nanoparticles to improve the delivery of pirfenidone, an anti-fibrotic drug, in the ocular environment, and 2) to achieve delivery of pirfenidone to the posterior segment of the eye for the treatment of PVR in a penetrating eye injury rabbit model. To achieve the project objectives, the following three specific aims will be completed:

Specific Aim 1: Development, characterization, and optimization of RPE-targeting AFA-PLGA nanoparticles for the delivery of pirfenidone to the posterior segment of the eye.

STATUS: Several batches of pirfenidone-loaded nanoparticles have been fabricated and characterized (Y1Q4).

Specific Aim 2: Evaluation of pirfenidone-loaded RPE-targeting AFA-PLGA nanoparticles *in vitro* for determination of biocompatibility and pharmacodynamics of release.

STATUS: Pending. An *in vitro* model of PVR will be used to test pirfenidone-loaded PLGA nanoparticles for their biocompatibility and anti-fibrotic effects after completion of Specific Aim 1.

Specific Aim 3: Assessment of *in vivo* safety, pharmacokinetics, and bioefficacy of intravitreally injected pirfenidone-loaded RPE-targeting AFA-PLGA nanoparticles in a penetrating eye injury rabbit model.

STATUS: Animal protocol for the *in vivo* study has been drafted (Y1Q4), pending review and submission to the IACUC for approval.

What was accomplished under these goals? (Detailed progress and results.)

Specific Aim 1: Development, characterization, and optimization of RPE-targeting AFA-PLGA nanoparticles for the delivery of pirfenidone to the posterior segment of the eye.

Key Findings or Accomplishments:

1. PLGA nanoparticles were fabricated via a modified nanoprecipitation method. Briefly, PLGA solution in tetrahydrofuran (THF) was added to Pluronic PF68/PF127 solution to form a milky white suspension. The organic solvent was evaporated in room temperature overnight. The polymer suspension was subjected to centrifugation at 10,000 RPM for 10 min to separate the particles from Pluronic PF68/F127. After several washes and resuspension in DI water, the resulting dispersion was freeze-dried to obtain dry particles of PLGA (blank PLGA nanoparticles). Pirfenidone was added to the organic phase to synthesize pirfenidone-loaded nanoparticles using the same method.
2. Morphological characterization of blank or pirfenidone-loaded PLGA nanoparticles was conducted using scanning electron microscopy (SEM). Briefly, the SEM samples were prepared by dispersing nanoparticles in DI water and placing a drop of the suspension on a piece of aluminum foil. The

aluminum foil was mounted on a copper stub and subjected to gold-platinum sputter coating. The nanoparticle samples were observed and imaged at 25,000X magnification.

3. Size distribution and zeta potential analysis were performed. PLGA nanoparticles were examined for size homogeneity using a Zetasizer Nano ZS90 (Malvern Instrument, Malvern, UK), a particle size analyzer with a detectable size range between 1 nm to 3 µm. Zeta potential (electrophoretic mobility) of the nanoparticles was also analyzed according to the manufacturer's instructions. The nanoparticles were dispersed in DI water and the analysis was performed at 25°C using a scattering angle of 90°.

Specific Aim 2: Evaluation of pirfenidone-loaded RPE-targeting AFA-PLGA nanoparticles *in vitro* for determination of biocompatibility and pharmacodynamics of release.

Key Findings or Accomplishments: N/A.

Specific Aim 3: Assessment of *in vivo* safety, pharmacokinetics, and bioefficacy of intravitreally injected pirfenidone-loaded RPE-targeting AFA-PLGA nanoparticles in a penetrating eye injury rabbit model.

Key Findings or Accomplishments: N/A.

What opportunities for training and professional development has the project provided?

Nothing to report for now.

How were the results disseminated to communities of interest?

We published a manuscript on the animal model that will be used to test the efficacy of pirfenidone-loaded nanoparticles and other therapeutics for retinal fibrosis (Mil Med. 2020 Jan 7; 185 (Suppl 1): 443-447).

Plans for the next reporting period to accomplish the goals

We will continue to develop pirfenidone-loaded nanoparticles and determine the loading efficiency/capacity of these particles. Optimization for drug encapsulation and delivery will also be performed.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report now since we are in an early stage of the project. However, at the completion of the proposed research, invaluable data will be generated in the development of novel biodegradable PLGA nanoparticles that can enhance the bioavailability of pirfenidone, facilitate its delivery to the posterior segment of the eye and reduce fibrosis in rabbits with penetrating eye injury. The impact of these studies will be significant for several reasons. As of now, an effective treatment for PVR is lacking. Even with surgical intervention, the visual outcomes for patients are poor leading to severe visual impairment or blindness. Furthermore, an effective system for delivering pharmaceutical agents to the posterior segment is not currently available.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. **CHANGES/PROBLEMS**

IMPORTANT REMINDER – Award recipient organization is required to obtain prior written approval from the awarding agency Contracting/Grants Officer whenever there are significant changes in the project or its direction such as significant change in scope or the Statement of Work (e.g. removal, change, or addition of aims/tasks or animal model change), change in PI or key personnel, reduction of 25% FTE, or significant change in budget.

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

The acquisition of funds was delayed due to a change in the principal performing site from the USAISR to NAMRU-SA. A budget modification was finalized on May 14, 2020. A CRADA between NAMRU-SA and METIS foundation was approved on June 3, 2020. Furthermore, the COVID-19 pandemic has shuttered our laboratories and research facilities. JBSA has been on HPCON Charlie causing all personnel to be on full time telework since March 2020. This caused significant delays in the synthesis of nanoparticles for this project. A plan for a safe return to work has been laid out and will be implemented when JBSA resumes normal operations under HPCON B.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

TOTAL PROTOCOL(S): 1

PROTOCOL (X of Y total):
IACUC Protocol Number: Not available.
ACURO Protocol Number:
Protocol PI:
Protocol Site: USAISR
Protocol Title: Write Title
Number of Animals Approved for Use: Write Number
IACUC INITIAL APPROVAL DATE: M/D/YYYY (expires M/D/YYYY)
ACURO INITIAL APPROVAL DATE: M/D/YYYY
RENEWAL APPROVAL DATES:
- Due M/D/YYYY

AMENDMENTS:
- None.

ADVERSE EVENTS OR UNANTICIPATED PROBLEMS:
- None.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS

Journal publications

1. Greene W, Burke T, Bramblett G, **Wang HC**. Detection of Retinal Fibrosis in a Rabbit Model of Penetrating Eye Injury. Mil Med. 2020 Jan 7; 185 (Suppl 1): 443-447. doi: 10.1093/milmed/usz221. Pubmed PMID: 32074329.
    a. Original manuscript
    b. Published
    c. Directly related to SOW, Specific Aim 3
    d. DoD funding acknowledged

Books or other non-periodical, one-time publications

Nothing to Report.

Other publications, conference papers, and presentations

Nothing to Report.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Naval Medical Research Unit-San Antonio (NAMRU-SA)
Name: Dr. Heuy-Ching Wang
Project Role: Principal Investigator
Nearest person month worked: 2 months
Contribution to the project: Dr. Wang has been involved in the budget modification, preparation and submission of the cooperative research and development agreement (CRADA) between NAMRU-SA and METIS foundation, in addition to setting up the laboratory for the proposed project.

Name: Dr. Zakiya Skeete
Project Role: Investigator
Nearest person month work: 3 months
Contribution to the project: Dr. Skeete has written the laboratory protocol for the synthesis of nanoparticles.

Name: Dr. Christina Rettinger
Project Role: Investigator
Nearest person month work: 1 month
Contribution to the project: Dr. Rettinger has drafted an animal protocol for testing the therapeutic effects of pirfenidone-loaded nanoparticles.

Name: Dr. Binapani Mahaling
Project Role: Investigator
Nearest person month work: 1 month
Contribution to the project: Dr. Mahaling has been involved in the synthesis and characterization of pirfenidone -loaded nanoparticles.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHART
Convert this report to a PDF file and append updated quarterly Quad Chart in PDF as an appendix.

9. APPENDICES

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Development of Nanopharmaceutical Therapy for Combat-Related Proliferative Vitreoretinopathy

Log#BA190144 W81XWH1920052

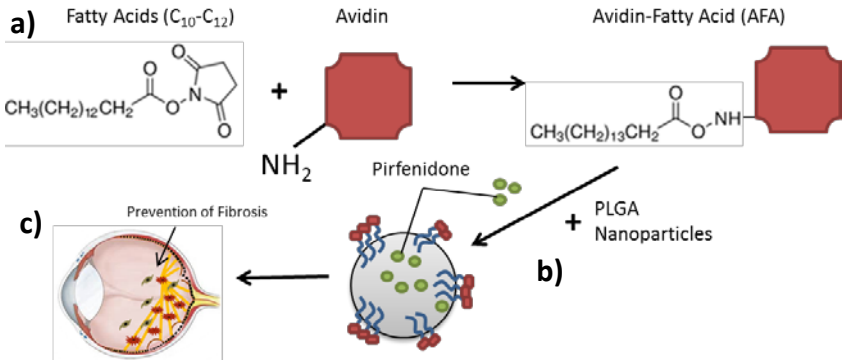
PI: Heuy-Ching H Wang, Ph.D. ***Org:*** *The Metis Foundation* ***Award amount:*** $2,449,953

Study Aims:

- Develop, characterize, and optimize of RPE-specific pirfenidone-loaded AFA-PLGA nanoparticles for delivery to the posterior ocular segment.
- Evaluate pirfenidone-loaded AFA-PLGA Nanoparticle *in vitro* for the determination of biocompatibility and pharmacodynamics of release.
- Assess *in vivo* safety, pharmacokinetics, and bioefficacy of intravitreally injected pirfenidone-loaded AFA-PLGA nanoparticles in an animal model of penetrating eye injury.

Approach:

Proliferative vitreoretinopathy (PVR), a type of intraocular fibrosis that develops after penetrating eye injury, leads to loss of vision. Pirfenidone, an FDA-approved anti-fibrotic drug, will be loaded into functionalized PLGA nanoparticles to enable delivery to the posterior ocular segment. Pirfenidone-loaded nanoparticles will be analyzed for safety, improved drug delivery, and prevention of intraocular fibrosis *in vitro* and *in vivo* in an animal model of penetrating eye injury.



a) RPE-Specific Avidin-Fatty Acid Functionalization Conjugate.

b) Fabrication of Functionalized Pirfenidone-loaded AFA-PLGA nanoparticles.

c) Application of Pirfenidone-loaded AFA-PLGA for the prevention of fibrosis.

TIMELINE & COST

Activities FY	20	20-21	21-22
Development, characterization, and optimization of RPE-specific pirfenidone-loaded AFA-PLGA nanoparticles	→	→	→
Evaluation of pirfenidone-loaded AFA-PLGA Nanoparticle in vitro	→	→	
Assessment of *in vivo* safety, pharmacokinetics and bioefficacy of pirfenidone-loaded AFA-PLGA nanoparticles		→	→
Manuscript preparation and Patent Submissions		→ →	→
Estimated Budget ($K)	$613K	$876K	$960K

Oct 14, 2020

Goals

CY20 Goal – Synthesis of Pirfenidone-loaded nanoparticles

- ☐ Optimize drug encapsulation in AFA-PLGA nanoparticles
- ☐ Drug release kinetic assays
- ☐ Cytotoxicity assays
- ☐ Generate stable cell line Pr-αSMA-Gluc RPE

CY20-21 Goals –Characterization of nanoparticles *in vitro*

- ☐ *In vitro* analysis of specificity and inhibition of fibrosis
- ☐ Obtain IACUAC approval
- ☐ Initiate *in vivo* studies

CY21-22 Goal – Characterize Pirfenidone-loaded nanoparticles *in vivo*.

- ☐ Complete characterization of Pirfenidone-loaded nanoparticles *in vitro*
- ☐ Continue *in vivo* studies
- ☐ Data analysis and manuscript preparation.

Comments/Challenges/Issues/Concerns

- ☐ Military personnel in combat zones are vulnerable to penetrating eye injuries
- ☐ PVR is an understudied and underfunded consequence of combat-related ocular trauma

Budget Expenditure to Date: September 15,2019-September 15,2020
Projected Expenditure for Years 01-03: $2,449,953
Actual Expenditure: $102,065.98

Detection of Retinal Fibrosis in a Rabbit Model of Penetrating Eye Injury

Whitney Greene, PhD; Teresa Burke, BS*; LTC Gregory Bramblett, MC, USA (Ret.)*; Heuy-Ching Wang, PhD**

ABSTRACT Introduction: To establish a rabbit model of posterior penetrating eye injury as a platform to test potential therapeutics. Materials and Methods: Anesthetized rabbits received posterior penetrating eye injury in one eye, whereas contralateral eyes were maintained as uninjured controls. Rabbits were randomized into two experimental groups. Group A was euthanized on Day 14 postinjury to determine retinal fibrosis at an early phase of disease progression. Group B was euthanized on Day 28 postinjury to examine retinal fibrosis at a late phase of disease progression. We examined animals on postinjury Days 7, 14, 21, and 28 with indirect ophthalmoscope and fundus photography. After euthanasia, eyes were processed for histology and immunofluorescence labeling of fibrotic proteins α-smooth muscle actin and collagen I. Results: Early fibrosis was detected by Day 14, as indicated by indirect ophthalmoscopy and fundus imaging. Fibrotic membranes were visible at sites of injury. Immunofluorescence analysis detected α-smooth muscle actin and collagen I within the fibrotic membranes. Conclusions: These data show that ocular fibrosis can be detected within 14 days after initial injury, with more severe fibrosis detected at 28 days postinjury. These results will be used to determine the optimal time points for later studies designed to test treatment strategies.

INTRODUCTION

Ocular injury is the fourth most common battlefield injury sustained in the Iraq and Afghanistan conflicts, behind amputation, traumatic brain injury, and posttraumatic stress disorder.[1] As weaponry and military tactics have advanced, the causes of eye injury have also changed. In recent military conflicts, explosive detonations accounted for 50% to 80% of eye injuries.[2,3] Without proper protection, the eye is especially vulnerable to secondary blast injuries caused by flying debris or fragments. Rapidly accelerating sharp particles can lacerate the cornea or sclera and enter the eye. Penetrating injuries by definition penetrate into the eye but not through and through—there is no exit wound. In severe cases, a sharp object penetrates to the posterior retina, wounds the tissue, and creates a posterior penetrating eye injury. After severe penetrating eye injury, tissues in the eye activate the wound healing process to repair the injury; however, wound healing in the eye often leads to fibrosis rather than functional repair and vision restoration. Intraocular fibrosis is characterized histologically by cell migration into the vitreous cavity, fibrocellular membrane production, and epiretinal membrane (ERM) formation.[4–7] The ERM attaches to the retina and contracts, causing tractional retinal detachment, which ultimately leads to vision loss. Despite technical advances in surgical management to remove the ERM and reattach the retina, the ERM will eventually reform and retinal detachment recurs.[8] The cycle of postinjury ERM/retinal detachment/surgery/ERM/retinal detachment is collectively known as proliferative vitreoretinopathy (PVR). Unfortunately, there are no effective therapeutics to prevent or treat this blinding disorder.

The patterns and characteristics of penetrating eye injuries are due to many variables in each individual injury. Since variables are more precisely controlled in a laboratory environment than in a random patient population, an experimental animal model of a posterior penetrating eye injury was developed. The objective of this study was to establish a reproducible model of posterior penetrating eye injury in the rabbit that will provide a platform to test effective potential therapeutics and methods of drug delivery. Several reports of animal models of PVR have been published; however, those models relied upon intravitreal injection of exogenous cells to induce ERM formation and retinal detachment.[9–11] Our model differs from previously published models because the injury mechanism is highly relevant to combat-induced eye injuries suffered by military personnel; therefore, the time to onset of intraocular fibrosis and cellular pathology will be similar to the pathogenesis of human PVR.

Modern adaptations in military tactics have led to changes in injury patterns sustained by combatants. According to one Operation Iraqi Freedom snapshot survey, approximately 16% to 17% of military medical evacuations were the result of eye injury.[2] Penetrating eye injury is considered to be a major risk

*Sensory Trauma, United States Army Institute of Surgical Research, 3698 Chambers Pass, Fort Sam Houston, TX 78234

Presented as poster at the 2018 Military Health System Research Symposium, Orlando, FL, August (Abstract number: MHSRS-18-0995); presented as an oral presentation at the American Academy of Optometry Meeting, San Antonio, TX, November 2018; presented as a poster at the Association for Research in Vision and Ophthalmology meeting, Honolulu, HI, May 2018.

The opinions or assertions contained herein are the private view of the author and are not be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

Animal Welfare Act Statement: Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility's Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by AAALAC International.

doi:10.1093/milmed/usz221

TABLE I. Experimental Design to Develop Rabbit Model of Penetrating Eye Injury

Group	Left Eye	Right Eye	Postprocedure Examination	Euthanasia Time Points	Total Number of Animals
A	Control	Penetrating injury	Days 0, 7,14	Day 14	5
B	Control	Penetrating injury	Days 0, 7, 14, 21, 28	Day 28	5

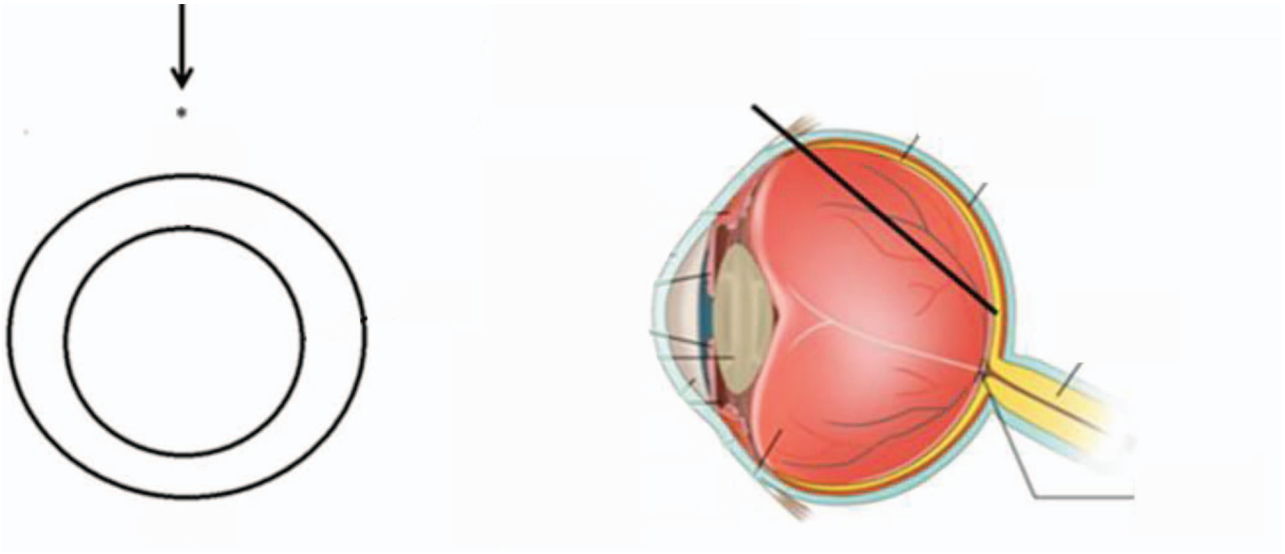


FIGURE 1. Needle insertion used to create posterior penetrating eye injury.

factor for the development of intraocular fibrosis. Studies of combat-related eye injuries suggest that intraocular fibrosis occurs after approximately 20% of closed-globe and 60% of open-globe injuries.[2,3] The incidence of intraocular fibrosis displays a bimodal distribution across age groups, with one peak representing patients between 65 and 69 years of age and the second peak representing patients between 20 and 29 years of age.[12] Consequently, intraocular fibrosis is one of the most frequent secondary outcomes in combat-related eye injuries.[2,3] Penetrating eye injuries associated with combat ocular trauma result in poor visual and anatomic outcomes despite surgical intervention.[5] Therefore, novel surgical and pharmacologic therapies are required to improve the functional and anatomic outcomes of these devastating injuries.

MATERIALS AND METHODS

Animal Care and Experimental Design

This study was conducted using male Dutch Belted rabbits (Covance, Princeton, New Jersey). Ten rabbits were divided into groups as listed in Table I. All animal procedures performed in this study adhere to the provisions of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the United States Army Institute of Surgical Research. To initiate this study, rabbits were anesthetized as described below and prescreened to determine the health of the eyes before proceeding with the baseline eye tests. Animals that passed the prescreening exam were assessed by fundus photography to document baseline (preinjury) morphology. Although still anesthetized, a posterior penetrating eye injury was induced in the right eye with the left eye acting as the control. Immediately following the injury procedure, the animals were examined by fundus photography to ensure that the injury has been induced correctly and that the globe did not rupture.

After induction of the eye injury, group A was euthanized on Day 14 to determine the degree of fibrosis at an early phase of disease progression. Group B was euthanized on Day 28 to examine the degree of fibrosis at a late phase of disease progression. In humans, ocular fibrosis usually manifests a month or longer after the initial injury; however, published experimental models with rabbits have shown that ocular fibrosis can be detected within 14 days after the initial injury.[13] In this model development study, rabbits were examined at both 14 and 28 days postinjury. The detection of intraocular fibrosis at any time point was considered successful development of this model.

Anesthesia

Preinjury analgesia was achieved with buprenorphine SR LAB at 0.5 mg/kg intramuscularly. The animals were anesthetized with ketamine 25 to 50 mg/kg and xylazine 2.5 to 10 mg/kg given intramuscularly (or subcutaneously). If additional anesthesia was required, a second dose of ketamine/xylazine

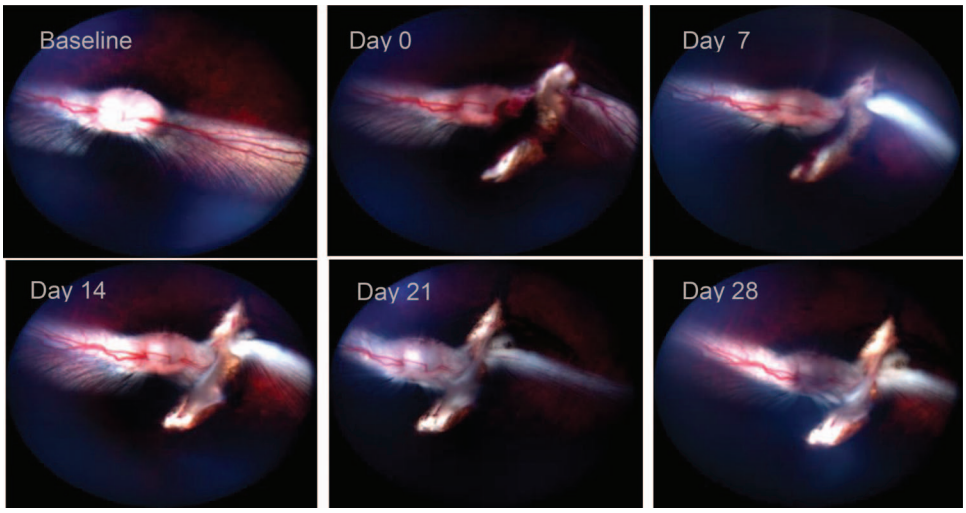


FIGURE 2. Fundus images depict development of intraocular fibrosis following penetrating eye injury.

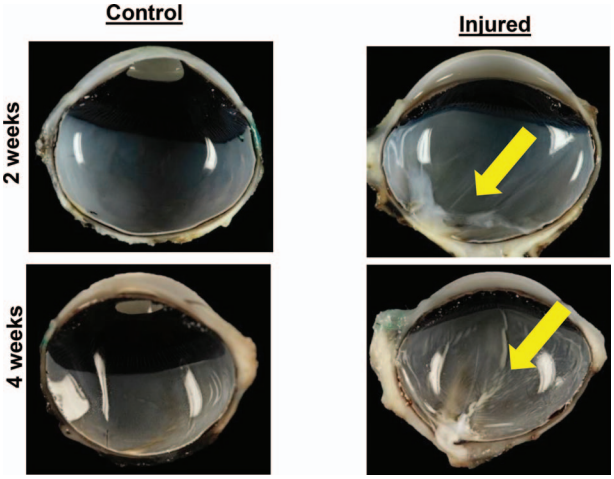


FIGURE 3. Radial sections of enucleated eyes. Control eye (left) with normal retinal attachment and injured eye (right) 14 and 28 days after penetrating eye injury with ERMs, retinal buckling, folds, and fibrosis as indicated by *arrows*.

mixture was administered at 25% to 50% of the original dose. As necessary, isoflurane anesthesia (1%–4%) delivered via face mask to effect was used in place of or in addition to injectable ketamine/xylazine anesthesia. At the indicated time point, rabbits were euthanized with an intracardiac dose of veterinary euthanasia solution (Fatal-Plus; 150 mg/kg) while under general anesthesia.

Induction of Penetrating Eye Injury

After the baseline examination, the right eye of each anesthetized rabbit was subjected to a penetrating injury. The eyes were washed once with 5% povidone iodide. Using calipers, a 23-gauge needle was positioned approximately 4 mm behind the superior limbus at the 12 o'clock position (Fig. 1). Under indirect ophthalmoscopic control, the needle was introduced into the vitreous cavity at approximately 0.5 in depth at a slight angle to avoid the lens, then turned at an angle to scratch the retina, returned to the inserting angle, and then removed. 0.5% moxifloxacin hydrochloride antibiotic drops were applied to the eyes immediately after the procedure. The contralateral uninjured eye of each animal was treated identically as the right eye as it acts as a control.

Fundus Imaging

Anesthetized rabbits were monitored using fundus photography (Phoenix Micron IV, Pleasanton, California) at the designated time points listed in Table I.

Tissue Processing and Immunohistochemistry

Rabbit eyes were harvested at the specified end points (Table I) for histological processing. After fixation in Davidson fixative for 24 hours, the eyes were switched to 10% neutral-buffered formalin then paraffin embedded, sectioned (5–7 μm slices), and stained with hematoxylin–eosin. Microscopic evaluation of the eyes was performed by a veterinary pathologist. Tissue sections were stained using established immunohistochemistry techniques to detect α-smooth muscle actin (α-SMA, ab7817; Abcam, Cambridge, Massachusetts) and collagen I (COLI, ab90395; Abcam) according to the manufacturer's directions. Alexa Fluor 488 goat antimouse IgG was used as a secondary antibody (A 11029; Thermo Fisher Scientific, Waltham, Massachusetts) to visualize labeled proteins. Cell nuclei were counter stained with blue fluorescent 4′,6-diamidino-2-phenylindole nuclei acid stain. Images were acquired using an Olympus (Center Valley, Pennsylvania) BX53 microscope equipped with a DP73 camera at ×10 magnification.

RESULTS

Anesthetized rabbits were monitored using fundus photography at baseline (preinjury) and at the designated time points

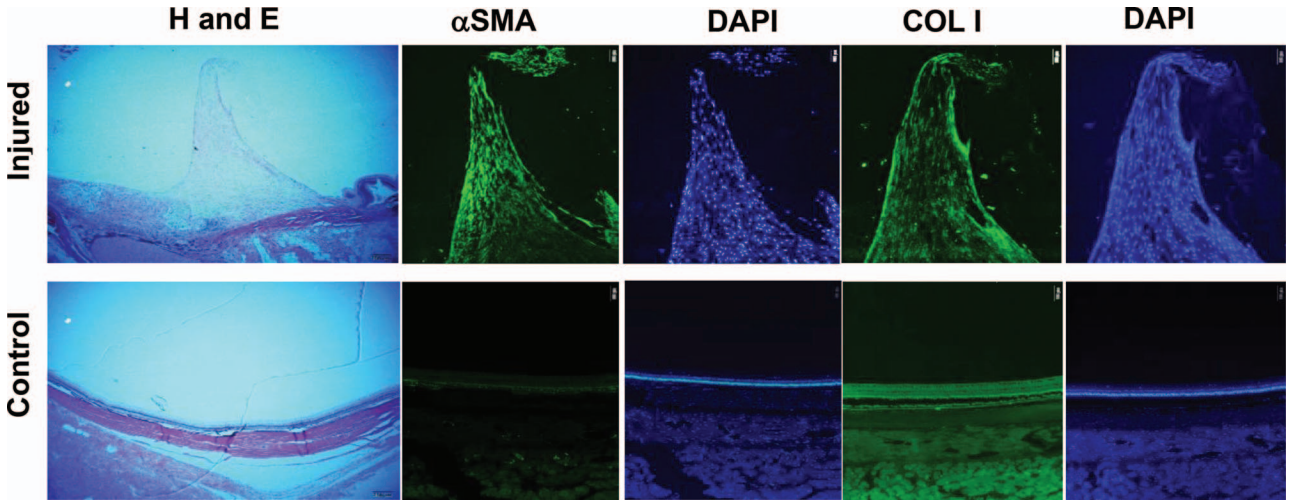


FIGURE 4. Serial tissue sections depicting the detection of α-smooth muscle actin (middle) and collagen I (right) fibrotic membranes at 28 days postpenetrating eye injury (top row) compared with the contralateral uninjured eye (bottom row).

listed in Table I. A fundus image from the normal uninjured eye is seen in Figure 2, compared with the fundus images from the eye immediately following injury and 7, 14, 21, and 28 days postinjury. At baseline, the retina, vasculature, and visual streak are normal and intact. Immediately after induction of the penetrating eye injury, the retina is visibly torn, and the vasculature is disrupted, and intravitreal hemorrhaging is observed. By Day 7 postinjury, intravitreal hemorrhage has subsided, whereas the vasculature to the right of the injury is ischemic. By Day 14 postinjury, ischemia is still observed to the right of the injury, and the injury begins to show evidence of fibrosis. By Days 21 and 28 postinjury, both fibrotic tissue and ischemia remain evident. After Day 14 or 28, the eyes were harvested and processed as described. The processed eyes were cross sectioned to examine the interior morphology. A diagnostic characteristic of intraocular fibrosis is ERM formation within the vitreous cavity after retinal tears or detachments.[4–7,13,14] As shown in Figure 3, ERMs are visible within 14 days postinjury, whereas by Day 28, retinal buckling, folds, and fibrosis (as indicated by arrows) indicate more severe intraocular fibrosis. The eyes were then paraffin embedded to enable the cutting of tissue sections for immunohistochemistry and immunofluorescence to detect markers or fibrosis and inflammation. The panel of photomicrographs shown in Figure 4 depicts serial tissue sections from an injured eye (top) and a normal control eye (bottom). The first two images at the far left depict hematoxylin- and eosin-stained images in which an ERM extending into the vitreous cavity is visible in the injured eye (arrow). The next two columns of images depict tissue sections from the same eyes that were labeled to visualize the fibrotic protein α-SMA (green) and nuclei (blue). The last two columns depict images of tissue sections that were stained to visualize COLI (green) another protein marker of fibrosis and nuclei (blue). Both α-SMA and COLI were detected in the ERM of the injured eye.

CONCLUSIONS

These data have shown that ocular fibrosis can be detected within 14 days after the initial injury, with more severe fibrosis detected at 28 days postinjury. The detection of intraocular fibrosis at any time point was considered successful development of this model. α-SMA and COLI are two markers of fibrosis. α-SMA, an important mediator of contractibility that induces retinal buckling and secondary detachment, was found to be highly expressed in the ERMs of the injured eyes. Although ischemia has not been reported as a risk factor for development of PVR, animals that exhibited postinjury ischemia developed fibrosis more quickly and had more severe PVR symptoms, including larger ERMs and increased retinal buckling. The contribution of ischemia to PVR pathogenesis is an interesting finding that will be further investigated in future studies. In total, these results confirm that this rabbit model of penetrating eye injury closely recapitulates the pathology of disease progression that occurs in humans with penetrating eye injury. This model provides a valid platform for testing potential therapeutics to treat intraocular fibrosis in humans, including injured service members who are at high risk to develop this blinding disorder.

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