ABSTRACT

Retinal injury due to excessive light exposure during military duties often results in serious vision damage to soldiers including irreversible loss of visual function. However, therapeutic interventions that can promote retinal protection or reverse retinal damage are very limited. This unmet clinical need also persists in the public when strong lasers, light, or fire cause trauma in ocular tissues. It is well known that estrogen has been shown to exhibit various beneficial actions in the central nervous system, including positively affecting mood and protecting the neuronal cells against neurodegenerative diseases. Despite estrogen's potential, its detrimental side effects prevent its clinical uses for neurotherapy.

To overcome this challenge, a bioprecursor prodrug was developed, called 10β,17β-dihydroxyestra-1,4-dien-3-one (DHED), that is selectively converted to E2 only in the neuronal cells, including retinal cells. To determine if treatment with DHED can sufficiently protect the photoreceptor cells from light-induced damage, male C57BL/6J mice were injected with or without 100 μg/kg DHED (n=15), 200 μg/kg DHED (n=15), 400 μg/kg DHED (n=15) and 200 μg/kg E2 (n=15) for 10 days before the light injury. Seven days after the light exposure, the visual function and retinal structure were examined by the spectral-domain optical coherence tomography (SD-OCT) and electroretinogram (ERG).

After light exposure, we found massive photoreceptor loss as indicated by thinning of the outer nuclear layer (ONL) in groups that received no treatment. However, DHED treatment significantly prevented light-induced retinal structural changes and light-induced a- and b-wave reduction. Additionally, photoreceptor loss was decreased as indicated by increased outer

nucellar layer thickness and SD-OCT data. The photoreceptor protective effects upon DHED-derived E2 treatment are stronger than that of the direct E2 treatment, consistent with our earlier observation that targeted E2 delivery via DHED prodrug produces more robust neuroprotection than direct administration of E2.

In conclusion, our study supported our hypothesis that DHED is an efficacious and safe site-specific delivery agent to produce robust estrogen-mediated retinal neuroprotection.

RETINA-TARGETED ESTROGEN PRODRUG: A NEW

CONCEPT FOR RETINAL PROTECTION

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RETINA-TARGETED ESTROGEN PRODRUG: A NEW CONCEPT FOR RETINAL PROTECTION

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the

School of Biomedical Sciences at

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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Fort Worth, Texas

May 2022

ACKNOWLEDGMENTS

I would like to first thank my major professor, Dr. Hongli Wu, for her guidance and mentorship throughout this past year. Through our conversations, she has helped me understand how to become a professional and how to excel in both academics and life. She has imparted to me a tremendous amount of scientific knowledge and research skills that I will carry with me throughout my career. I also want to thank Dr. Yu Yu and Jinmin Zhang for all the support they provided to complete this project. Their expertise was especially significant with data collection and helping me understand complex lab procedures. They were always willing to help me even though they were extremely busy.

I also want to thank my committee members, Dr. Katalin Prokai-Tatrai and Dr. Yang Liu, for all their time, effort, and guidance this past year. They always offered excellent constructive critiques that helped me become a better research student. I am confident that the lessons I learned will be beneficial in the future.

Lastly, I would like to thank the School of Biomedical Sciences at the University of North Texas Health Science Center for this opportunity to attain my master's degree.

This research project was funded by the Department of Defense.

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CHAPTER I

BACKGROUND & LITERATURE REVIEW

The retina plays the function of converting light stimuli from the environment into neural signals which allow the brain to visually perceive our surroundings [12]. Considering its role, dysfunction of the retina can significantly impact the quality of an individual's life [21]. The retina is equipped with self-repair mechanisms that will prevent tissue damage under normal environmental conditions [22,23]. However, high intensity visual or infrared light from fires, lasers or other bright light can cause irreversible tissue damage in the form of thinning of the photoreceptors and outer nuclear layers which can act as precursors to age-related macular degeneration (AMD) [13,24]. Other risk factors for AMD include cardiovascular health, hormonal imbalance, genetics, and age [26]. Additionally, factors such as diet, smoking, genetics and aging are all considered risk factors [28].

There are two types of AMD: dry AMD (atrophic form) and wet AMD (neovascular form) [28,29]. The most common form of AMD is dry AMD where insoluble extracellular aggregates accumulate known as drusen. The latter stage of dry AMD is known as geographic atrophy where degeneration of the retinal pigment epithelium occurs [28]. At advanced stages, dry AMD can progress to wet AMD where new blood vessels form which is known as choroidal neovascularization. These blood vessels are weak and are heavily prone to leak their internal fluid into the surrounding tissues which can lead to scarring and blindness [27,28,29].

Treatment for wet AMD has been long established with effective drugs (Bevacizumab, Aflibercept, Ranibizumab) that disrupt angiogenesis by utilizing monoclonal antibodies to block vascular endothelial growth factors [27]. Although such drugs have been beneficial, they are unable to prevent or treat the initial onset of AMD and are designed only to relieve the impact caused by advanced wet AMD [27]. Additionally, in some cases, patients might have dry AMD for a prolonged period of time to which there are no existing treatments [27,28,29].

17β-estradiol (E2) is an essential hormone for both males and females. In addition to its important functions such as the development of the female reproductive systems and secondary characteristics, E2 also exhibits beneficial functions in the central nervous system. These actions include positively affecting mood and protecting neuronal cells against neurodegenerative diseases [31-39]. Additionally, estrogen receptors have been identified in the eye and studies have shown that the hormone displays strong neuroprotective effects on the retina [1,14]. Epidemiologic studies also show that women who experience early menopause have a higher risk of developing retinal degeneration [15]. In contrast, women who consume estrogen through hormonal therapies or oral contraceptives have a reduced risk of retinal degeneration compared to those who didn't have hormonal therapy [40]. Animal model studies show that estrogen can protect the photoreceptor from light-induced damage in both female OVX and male rodents [16-19]. Lastly, clinical evidence has shown exogenous estrogen offers protection against large drusen accumulation and depigmentation of the retinal pigment epithelium (RPE), both common features of AMD [20]. All evidence establishes estrogen as a potential therapeutic candidate for ocular neurotherapy and neuroprotection.

In a previous study using glaucoma models, it was shown that topical application of 17β-estradiol (E2) provided neuroprotection for the retinal ganglion cell (RGC) layers [1]. The axons of RGC cells comprise the optic nerve and communicate visual stimuli between the eye and brain. Glaucoma causes the destruction of these cells due to increased intraocular pressure (IOP) leading to vision loss [2]. The study showed that there was approximately a 50% reduction in RGC cell layer death when administering E2 to ovariectomized (OVX) rats [1]. Measurement of E2 using LC-MS/MS assay showed an increase in the hormone in the retina after treatment and provided significant neuroprotection. The study also showed that estrogen-regulated proteins were altered with E2 treatment that are significant for a healthy nervous system and proper functions [3]. However, the eye drop treatment also caused an increase in circulating E2 levels which would lead to adverse side effects associated with estrogen [1].

Systemic exposure to the estrogen hormone can cause elevated blood hormone levels and results in detrimental side effects which can limit its use in clinical settings. These side effects include breast and ovarian cancer, thrombosis, myocardial infarction, and stroke [4,5,6]. Additionally, the feminizing effects of estrogen also create a limitation that creates gender-specific restrictions [7]. Concomitantly these side effects create the need for a targeted estrogen therapy that can localize specifically to the site of action without systemic exposure.

To combat the limited utility of estrogen as a neuroprotectant, a new prodrug has been developed called 10β,17β-dihydroxyestra-1,4-dien-3-one (DHED). DHED remains inert and is converted to E2 selectively in the neuronal cells of the CNS even under systemic administration [8,9]. DHED is the substrate for short-chain NADPH-dependent dehydrogenase/reductase (SDR), an enzyme that is selectively present in neuronal cells of the CNS and catalyzes the production

of E2. Results from stroke model studies in rats showed that DHED treatment protected menopausal female rodents from symptoms of estrogen deprivation and offered neuroprotection through targeted E2 delivery [8]. This was accomplished without increasing systemic circulating E2 thus preventing the proliferation of cancerous tissue and other uterotrophic side effects. Additionally, the effects of DHED on animal models with Alzheimer's disease were examined and the data showed additional therapeutic benefits [10]. OVX and intact females were treated with vehicle, E2 or DHED treatments and it was observed that DHED treatment caused a decrease in amyloid precursor protein levels without increasing uterine weight. Most recently, a study explored the potential use of DHED-derived E2 as an ocular neuroprotectant in glaucoma model studies [51]. The data confirmed that DHED was able to penetrate the cornea and offer retinal selective E2 delivery without affecting peripheral tissues [51].

Considering the beneficial effects of DHED-derived E2 on the CNS tissues and the retina, we hope to learn more about its therapeutic implications as it pertains to photoreceptor degeneration in the retina. The retina is considered as an extension of the CNS and shares characteristics of CNS pathologies and estrogen receptors have also been identified in the eye [11,14]. As such, we hope to learn if DHED can be used to combat neurodegenerative diseases that cause photoreceptor loss seen in AMD.

In this study, we hope to show that DHED can be converted to E2 in the eye to prevent retinal damage without causing additional systemic side effects.

CHAPTER II

RESEARCH PROJECT

Specific Aims

Specific Aim 1: To confirm if DHED-derived E2 will offer protection for the function of the retina utilizing a light-induced retinal damage model. This aim will be accomplished by using a phototoxicity model to examine the potential of DHED-derived E2 in preventing photoreceptor cell death. Retinal damage will be induced through light exposure and DHED prodrug will be administered in varying concentrations. This aim will be achieved using an electroretinogram (ERG) and the retinal function of mice treated with DHED and E2 treatments will be compared. **Hypothesis:** E2 produced from the DHED prodrug will protect the photoreceptors from light-induced damage and limit the damage to retinal function.

Specific Aim 2: To determine the neuroprotective effect of DHED-derived E2 on the structure of the retina in a light-induced retinal damage model. We will utilize spectral-domain optical coherence tomography (SD-OCT) and fundus camera to visualize the retina and assess structural damage. We will use the SD-OCT to measure the outer nuclear layer (ONL) thickness of the retina to compare damage between different experimental groups. Additionally, we will also utilize the fundus camera to visualize the retina. **Hypothesis:** DHED-derived E2 will offer robust neuroprotection to retinal structures by delivering estrogen to the ocular tissues.

Significance & Innovation

Approximately 67 million people are currently affected with AMD in the European Union and the prevalence is projected to increase by 15% by 2050 due to the aging population [25,26]. In the United States alone there are approximately 11 million individuals affected with AMD and projections show an increase to 22 million by 2050 [30]. AMD is one of the leading causes of blindness in both industrialized and developing countries and its impact will only continue to increase [30]. Considering its widespread impacts, both preventive and therapeutic treatments are necessary that can be used in clinical settings effectively. Studies have shown that E2 can act as a neuroprotectant and prevent the effects of AMD [1,14,15,40]. However, the adverse effects of systemic administration of E2 limit its use in clinical settings [4-7].

One possible approach to solving this issue is by using the innovative technology of prodrugs. There are two types of prodrugs: carrier-linked prodrugs and bioprecursor prodrugs [41]. As the name implies, carrier-linked prodrugs require a transporter/carrier that is connected to the active molecule. After converting to the parent/active drug, the carrier group must be removed from the body [41,44]. In contrast, bioprecursor prodrugs, such as DHED, do not have a transporter (also termed promoiety) group and instead undergo metabolic activation through oxidation or reduction reactions to convert to the active drug [1,41,44]. This method allows for the creation of a new compound that can act as substrates for enzymes in tissues [41]. DHED is converted to E2 through hydride transfer from NADPH coenzyme. Subsequently, a spontaneous water elimination will occur that will cause the hydroxyl group to exit. The overall reaction is shown in figure 1. [1]

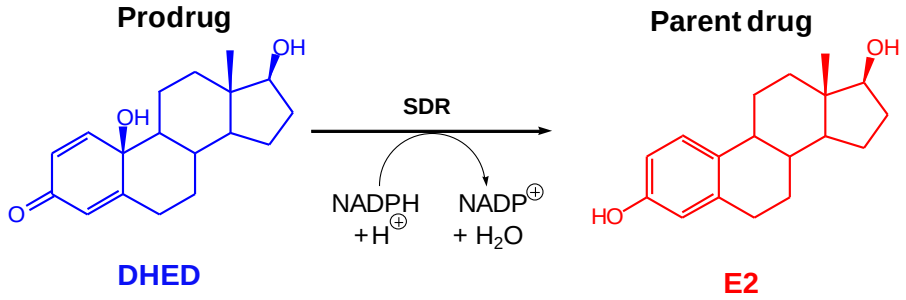

Figure 1. Schematic illustration of DHED prodrug conversion to the parent E2 in the retina by a NADPH-dependent short-chain dehydrogenase/reductase (SDR)

The term prodrug was coined by Adrien Albert in 1958 and can be summarized broadly as inactive derivatives of therapeutic agents that undergo biotransformation to the active metabolite at the site of action [1,41-43]. The prodrug DHED behaves as such by converting to estrogen selectively in the central nervous system due to the presence of the SDR enzyme [1]. Previous studies have established that DHED is effective in selectively converting to estrogen in neuronal cells without systemic exposure [8-10]. Because the retina is considered as an extension of CNS, this study utilizes SDR enzymes that are selective to the neuronal cells to convert DHED to estrogen in the retina to offer protection for photoreceptor cells and prevent retinal degeneration [11].

Materials & Methods

In this study, we specifically chose the C57BL/6J mouse strain because of the Rpe65 Leu450Met variation that makes the retina highly resistant to light injury [45,46]. This is significant because other mice strains do not have injury-resistant retinas compared to human

retinas. We chose mice that were 10-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) based on previous publications [16,18,19]and other preliminary studies.

10-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) which do not have any history of estrogen treatments will be intraperitoneally (i.p.) injected with 100 µg/kg DHED (n=15), 200 µg/kg DHED (n=15), 400 µg/kg DHED (n=15), and 200 µg/kg E2 (n=15) for 10 days. Additionally, a negative control, positive control and a vehicle control group were used to control for confounding variables. In the negative control group, animals were not given an injection of E2 or DHED and no injury took place. In the positive control group, animals were not given an injection but were exposed to light injury. Lastly, in the vehicle control group, animals were given corn oil injection with light injury. An overall flowchart of the experimental procedures is shown in figure 2.

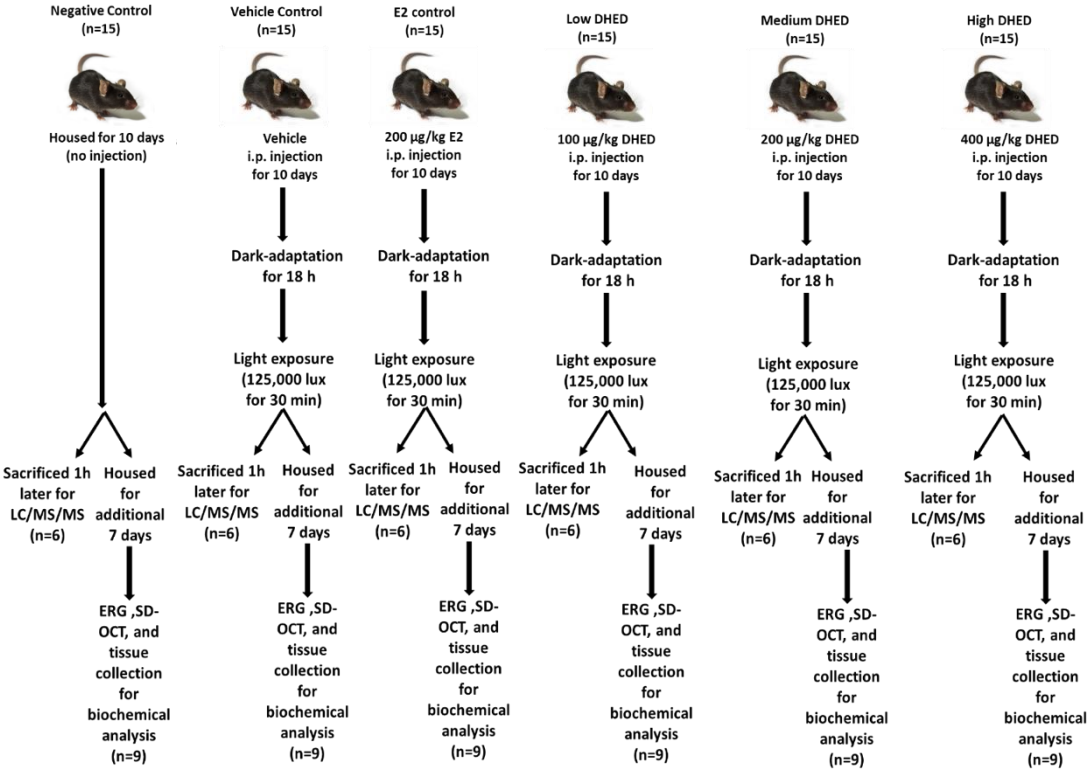

Figure 2. Experimental protocol

Electroretinography (ERG): ERG measures the function of the retina and is an established method to assess retinal health [47,48]. ERG analysis was performed at 7 days after light exposure by following protocols based on previous publications [45,46]. Seven days were chosen based on previous publications [19]. Before ERG, all mice were kept in a completely dark room overnight. After dark adaptation, the mice's eyes were dilated, and subsequently, the mice were sedated. Eyes were dilated using Phenylephrine HCl (2.5%) (Paragon BioTeck, Inc.) and Tropicamide (1%) (AKORN, Lake Forest, IL, USA) 15 minutes prior to visual testing. Mice were anesthetized with ketamine (100 mg/kg) (Vedco, St. Joseph, MO, USA) and xylazine (10 mg/kg) (Vedco) cocktail. EYE lubricant (Goniosoft, 2.5%, USA) was applied to the surface of each cornea to inhibit drying. A heating pad was placed under the body to maintain body temperature at approximately 36C. The ground electrode was a subcutaneous needle in the tail and the reference electrode was placed subcutaneously between the eyes. A corneal electrode was attached to the center of the cornea. ERG responses were recorded at increasing light intensities from -1.0 to 1.5 log cd sec./m^2 (**Ganzfeld System**). Responses ranging between 30 flashes with 2-sec intervals and 4 flashes with 10-sec intervals were averaged. Amplitudes and latencies of a-waves and b-waves were recorded. A-waves were measured from the baseline to the maximum peak of the a-wave and B-waves were measured from the trough of the a-wave to the peak of the b- wave.

Spectral-Domain Optical Coherence Tomography (SD-OCT) - To identify the photoreceptor degeneration, SD-OCT imaging was performed. Seven days after light exposure,

mouse retinas were imaged using Bioptigen Envisu^{TM} Ophthalmic Imaging System (Bioptigen, Durham, NC). Mice were anesthetized, and their pupils dilated as described previously in ERG protocols. Rectangular volume scans (1.2mm x 1.2mm) were performed. Each volume consisted of 1000 A-scans x 100 B-scans. The en-face image passing the center of the ON head was used for retinal thickness measurements. All four retinal quadrants (temporal, nasal, superior, and inferior) were imaged. Retinal layers were segmented and measured in each of the SD-OCT scans utilizing calipers in the InVivoVue^{TM} Clinic software (Bioptigen). ONL (Outer nuclear layer) thickness was measured from the interface of the ONL and OPL (outer plexiform layer) to the interface of the ONL and ELM (External limiting membrane). The total retinal thickness was measured as the distance from the interface of the nerve fiber layer and vitreous chamber to the interface of the RPE (Retinal pigment epithelium) and choroid.

Results

To fulfill the goals stated in specific aim 1, we used ERG to examine the function of the retina after light-induced retinal damage. The ERG data showed that DHED pretreatment prevented the decrease in the retinal function caused by retinal damage (Figure 3A and 3B). This was the case with both a-waves and b-waves which measure photoreceptor function and second-order neurons such as bipolar and muller cells, respectively [47,48]. The a-wave data showed a significant difference ($p < 0.05$) between control and estrogen treatment groups while no significant difference was seen between control and DHED treatment groups. The b-wave data showed a significant difference ($p <0.05$) between control and no treatment groups while no significant difference was seen between control and DHED treatment groups.

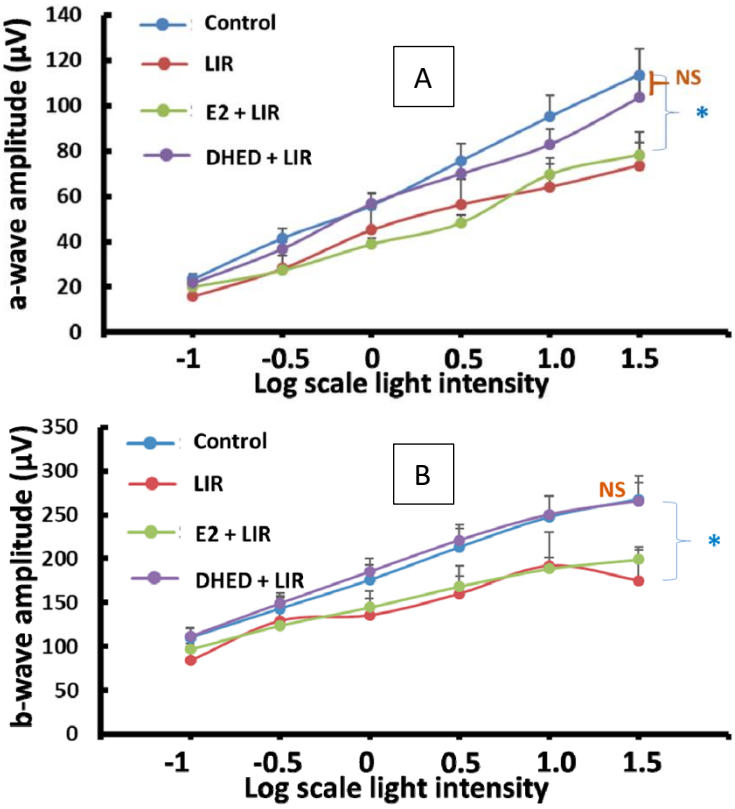

Figure 3A and 3B. DHED treatment (200 µg/kg) preserves the visual function as measured by ERG compared to E2 treatment (200 µg/kg). DHED prevented a- waves (A) and b-waves (B) reduction caused by light damage. * $P < 0.05$ (compared with control);

To further evaluate DHED's effect on the retina and fulfill specific aim 2, we utilized SD-OCT and fundus imaging to visualize the structure of the retina for each treatment group. The results from our preliminary data are shown in figure 4 and data from current experiment is shown in figure 5. Both tests showed visible damage and loss of photoreceptors to the controls that received no treatment such as positive and vehicle controls. The mice that had estrogen treatment also had some damage and photoreceptor loss but overall was better than the controls. The mice that received DHED treatment had minimal or no loss photoreceptors depending on DHED concentration.

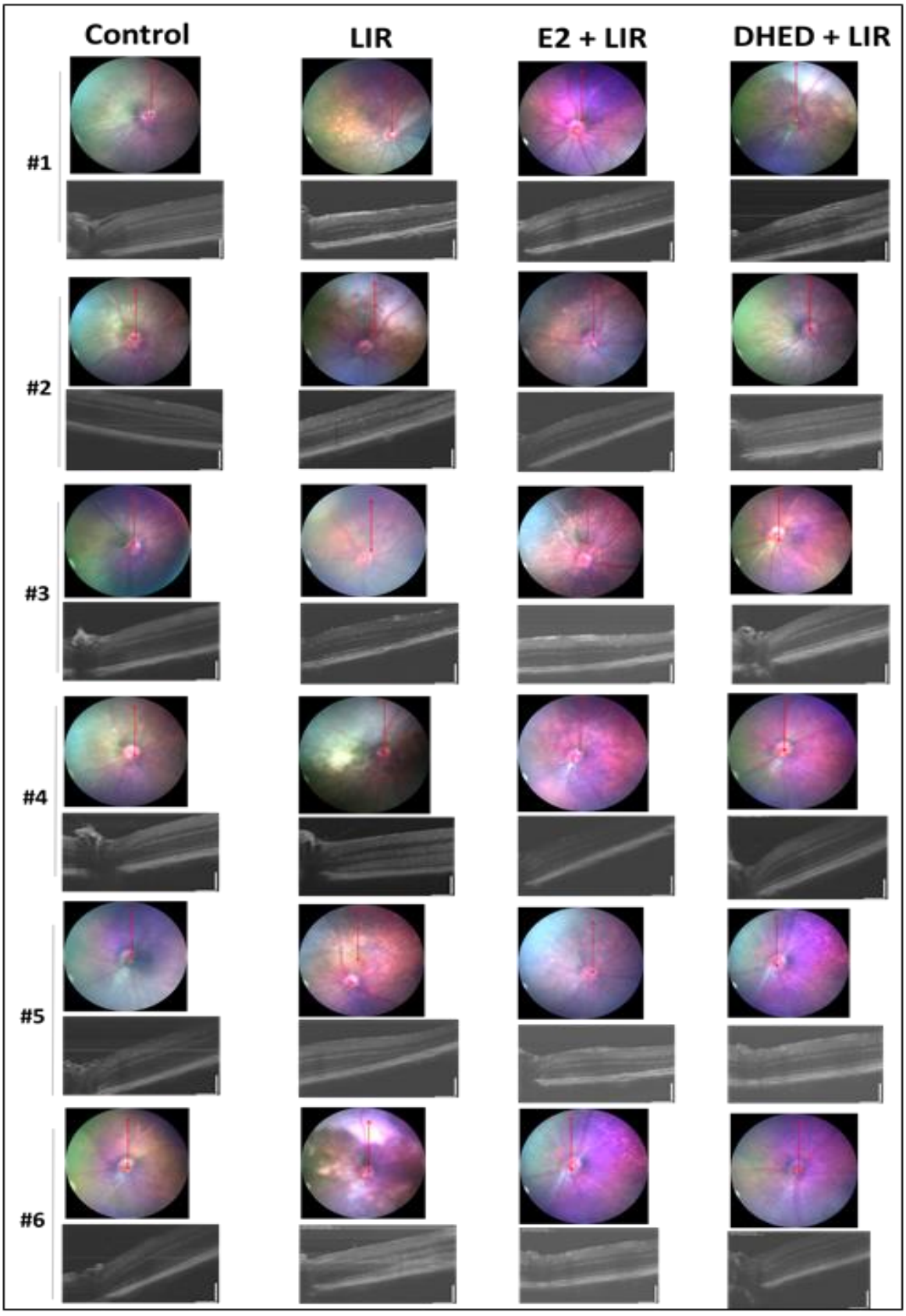

Figure 4. DHED and E2 treatments compared using SD-OCT and fundus camera

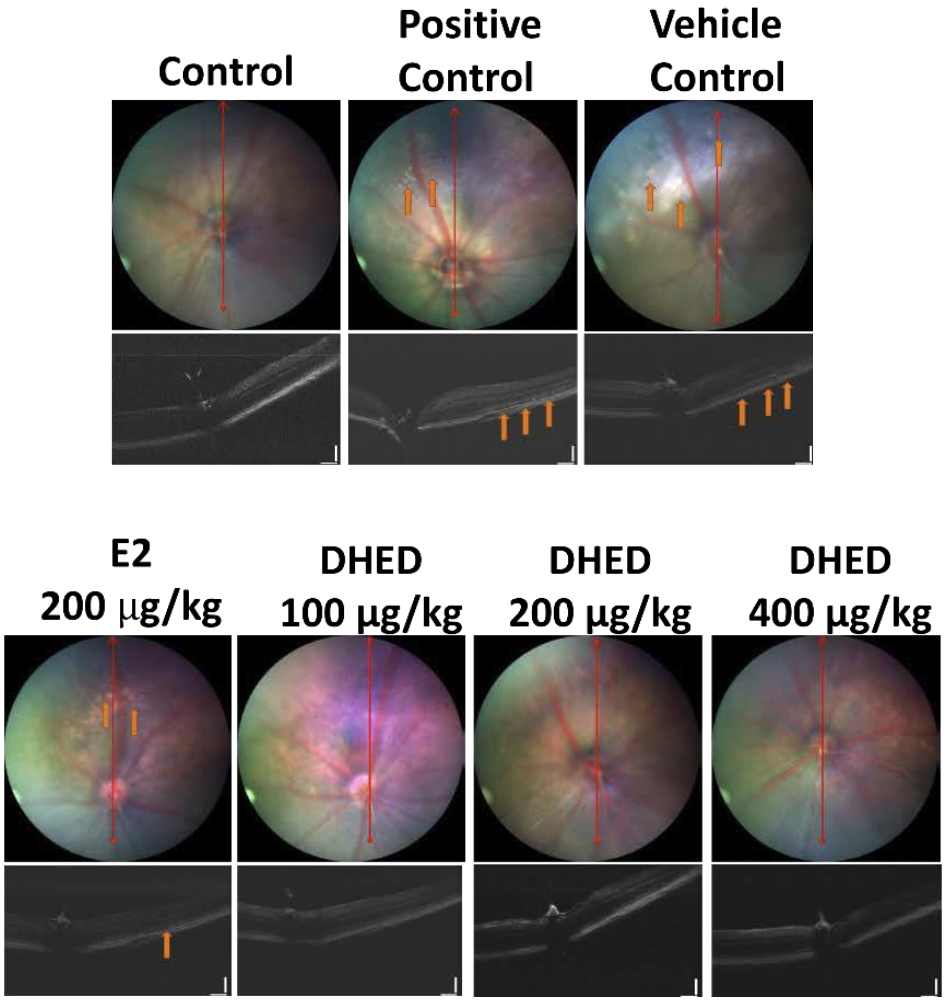

Figure 5. DHED-derived E2 protects the photoreceptors from light-induced damage. The eye exams fundus (upper panel) and SD-OCT (lower panel) were examined seven days after the light damage. DHED treatment produced more robust neuroprotection than direct E2 treatment.

To further confirm neuroprotective effects of estrogen through DHED prodrug, SD-OCT was used to access the thinning of the ONL (Outer nuclear layer) of the retina (Figure 6). As expected, positive and vehicle control groups had photoreceptor loss that thinned the ONL. In contrast, the E2 treatment and low DHED treatment group had a significantly thicker ONL compared to the controls ($p < 0.05$). Lastly, medium DHED and high DHED groups had a significantly thicker ONL compared to the positive control ($p < 0.001$) and E2 treatment group ($p < 0.01$). The high DHED group had the closest ONL thickness to the control group that had no light-induced retinal damage.

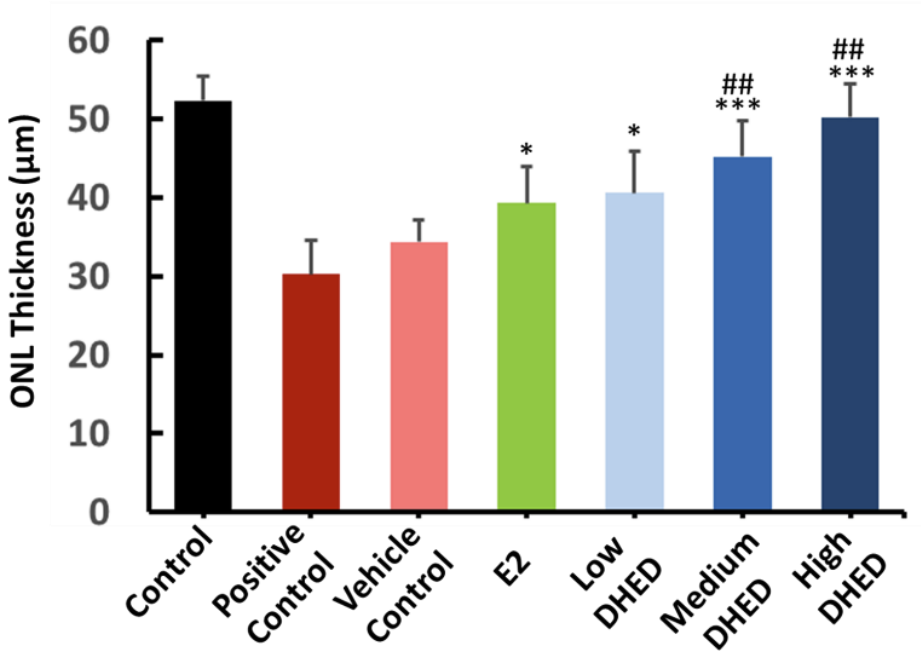

Figure 6. DHED-derived E2 preserves the ONL thickness from light-induced damage. *P<0.05, *** P <0.001 vs positive control; ## P<0.01 vs E2

Discussion

In this study, we examined the ability of DHED to deliver estrogen directly to retinal tissues to offer neuroprotection. This was accomplished through two specific aims. Specific aim 1: Confirm the neuroprotective effect of DHED-derived E2 on the structure of the retina in a light-induced retinal damage model. Specific aim 2: To determine if DHED-derived E2 can protect the function of the retina.

Estrogen has shown to be effective in combating retinal degeneration in various studies [1,14-19]. However, the challenge remains that peripheral exposure to estrogen causes adverse effects in both males and females. This restricts its use in the clinical setting as an effective treatment for AMD. DHED prodrug offers a solution to this dilemma using the prodrug technology to deliver the active drug directly to the site of action.

Our results from the ERG and SD-OCT data showed that DHED is effective in delivering estrogen and offers protection against retinal degeneration. In particular, DHED-derived E2 offered a more robust protection compared to direct E2 treatment. In ERG testing, DHED groups displayed better retention of function in both a-wave and b-wave data (Figure 3A and 3B). Likewise, in SD-OCT data, DHED treatment displayed better structural retention than E2 treatment groups by maintaining retinal thickness (Figure 4,5,6). There are multiple reasons for this occurrence. In comparison to the parent E2 molecule, DHED is much less lipophilic, more water-soluble and has decreased binding to plasma proteins [8,9,49,50]. A previous study showed that because of the aforementioned characteristics of DHED, approximately 10 times higher concentrations of E2 treatment would be needed to match the therapeutic effect of

DHED [9]. This balance of hydrophilicity and lipophilicity found in DHED is also ideal for corneal absorption [51] which is important for future developments of eye drops for patients.

DHED also offers a better “drug economy” compared to its parent molecule in E2 since it focuses the estrogen directly to the site of action. This is enabled by the prodrug technology that limits the systemic spread of estrogen that is seen in direct E2 treatments. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) based assays have shown that no peripheral exposure to estrogen is seen with DHED administration regardless of the route of administration (intravenous routes, eyedrops) [9,51,52]. The flexibility concerning the route of administration that DHED provides will allow for the development of a drug that is effective at the clinical stage. Additionally, the lack of peripheral exposure ensures that no adverse side effects will be present that are common in direct E2 treatments. Furthermore, proteomic studies have been done that examined protein expression comparing E2 and DHED treatments. It was observed that proteins that are up-regulated during direct E2 treatments, such as α-crystalline A chain (CRYAA) and β-crystalline B chain (CRYBB1), were also present with DHED treatment [53]. These proteins have characteristics similar to chaperones and heat shock proteins that help with resistance to apoptosis and prevent precipitation of denatured proteins which are essential during times of retinal degeneration [54-56].

There are currently some alternative treatments also being tested to combat retinal degeneration. It is established that light-induced damage occurs due to an increase in oxidative species [9]. Therefore, it could be beneficial to utilize natural and synthetic antioxidants such as L-stereoisomer of N-acetyl cysteine and N-nitro-arginine methyl ester among others [60]. However, it is unclear whether administration of antioxidants can effectively cross the blood-

retinal barrier which might be easier with DHED due to its ideal lipophilicity-hydrophilicity balance [51,60]. Other promising therapeutic studies include the use of ferrostatin-1 to inhibit ferroptosis and the use of monomethyl fumarate to activate Nrf2 transcription factor [61-63]. However, there is still a lot to learn about these novel therapeutic options and they are much less established compared to the use of estrogen as a neuroprotectant.

Limitations. The current protocol examines DHED capabilities from a preventive perspective but understanding whether DHED-derived E2 can be used as a treatment for retinal degeneration in future studies will be valuable. Additionally, this study only uses a single acute model of the light-induced retinal damage model. It is important to explore DHED in other models such as genetically inherited degeneration models like retinal degeneration 2 ($Prph2^{Rd2}$) and retinal degeneration 3 (rd3) among others [57-59]. Lastly, this study utilizes intraperitoneal administration of drugs which is not translatable to the clinical setting. Although the current stage in drug discovery is very early, it is important to explore more accessible drug administration through modes such as eyedrops or inhalation.

Future Directions. The results from this study display the promise DHED-derived E2 holds as a neuroprotectant to combat retinal degeneration. One possible future study could be to identify the lowest concentration of DHED required to offer robust neuroprotection. This would allow for better drug economy for future studies and provide drug developers with base point drug concentration that can be further explored. Additionally, our lab will also be utilizing LC-MS/MS bioassay to compare E2 and DHED treatments to understand the blood E2 distributions at different concentrations. We will analyze both ocular and non-ocular tissues such as kidney, liver, and seminal vesicles to confirm the lack of E2 exposure in DHED treatment

groups. This will ensure that the adverse side effects seen from direct E2 treatments will be avoided.

SUMMARY & CONCLUSIONS

In this study, we utilized a light-induced retinal damage model in mice to determine if DHED-derived E2 can act as a neuroprotectant and prevent retinal degeneration. We used SD-OCT, fundus camera and ERG to help gather necessary data after retinal injury. Overall, the data confirmed that DHED-derived E2 can act as a neuroprotectant to preserve retinal structure and function. Additionally, DHED treatment displayed a more robust protection than similar dosage E2 groups which further highlights the drug's benefits. DHED-derived E2 protection has been well established and utilizing the drug in other non-acute models will increase its credibility as a potent neuroprotectant. We hope that this data can be used in future studies as a base point to further explore the possibilities of utilizing DHED to combat retinal degeneration.

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