

DARPA Soldier Self Care: Rapid Healing of Laser Eye Injuries with Light Emitting Diode Technology

**Harry T. Whelan, M.D.¹, Margaret T. T. Wong-Riley, Ph.D.², Janis T. Eells, Ph.D.³,
James N. VerHoeve, Ph.D.⁴, Rina Das, Ph.D.⁵, Marti Jett, Ph.D.⁵**

¹Bleser Professor of Neurology
Medical College of Wisconsin
9000 W. Wisconsin Ave.
CHW OB #205
Milwaukee, WI 53226
Distinguished Research Professor
Center for Technology & National Security Policy
National Defense University
Washington, D.C. 20319

²Medical College of Wisconsin
Department of Cell Biology, Neurobiology and Anatomy
8701 Watertown Plank Road
Milwaukee, WI 53226

³University of Wisconsin-Milwaukee
College of Health Sciences
Milwaukee, Wisconsin 53201

⁴University of Wisconsin
Department of Ophthalmology
Madison, WI 53705

⁵Walter Reed Army Institute of Research
Washington D.C.

Abbreviations: LED, light-emitting diode; ERG, electroretinogram; LRRI, log relative retinal illumination; RGC, retinal ganglion cell; TTX, tetrodotoxin.

Photobiomodulation by light in the red to near infrared range (630-1000 nm) using low energy lasers or light-emitting diode (LED) arrays has been shown to accelerate wound healing, improve recovery from ischemic injury and attenuate degeneration in the injured optic nerve. At the cellular level, photoirradiation at low fluences can generate significant biological effects including cellular proliferation and the release of growth factors from cells. Mitochondrial cytochromes have been postulated as photoacceptors for red to near-infrared (NIR) light energy and reactive oxygen species or mitochondrial redox changes have been advanced as potential mediators of the biological effects of this light.

We hypothesize that the therapeutic effects of red to near infrared light result, in part, from intracellular signaling mechanisms triggered by the interaction of NIR light with the mitochondrial photoacceptor molecule cytochrome oxidase which culminate in improved cellular mitochondrial energy metabolism and

Paper presented at the RTO HFM Symposium on "Combat Casualty Care in Ground Based Tactical Situations: Trauma Technology and Emergency Medical Procedures", held in St. Pete Beach, USA, 16-18 August 2004, and published in RTO-MP-HFM-109.

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE 01 SEP 2004	2. REPORT TYPE N/A	3. DATES COVERED -	
4. TITLE AND SUBTITLE DARPA Soldier Self Care: Rapid Healing of Laser Eye Injuries with Light Emitting Diode Technology		5a. CONTRACT NUMBER	
		5b. GRANT NUMBER	
		5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)		5d. PROJECT NUMBER	
		5e. TASK NUMBER	
		5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Bleser Professor of Neurology Medical College of Wisconsin 9000 W. Wisconsin Ave. CHW OB #205 Milwaukee, WI 53226; Distinguished Research Professor Center for Technology & National Security Policy National Defense University Washington, D.C. 20319		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)	
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited			
13. SUPPLEMENTARY NOTES See also ADM001795, Combat Casualty Care in Ground-Based Tactical Situations: Trauma Technology and Emergency Medical Procedures (Soins aux blessés au combat dans des situations tactiques : echnologies des traumatismes et procédures médicales d'urgence)., The original document contains color images.			
14. ABSTRACT			
15. SUBJECT TERMS			
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified	UU
			18. NUMBER OF PAGES 18
			19a. NAME OF RESPONSIBLE PERSON

antioxidant production. In support of this hypothesis, we have demonstrated in primary neuronal cells that NIR-LED photo-irradiation (670 nm at 4 J/cm²) increases the production of cytochrome oxidase in cultured primary neurons, reverses the reduction of cytochrome oxidase activity produced by metabolic inhibitors and attenuates cyanide-induced apoptosis. We have also shown that the action spectrum of NIR light for stimulation of cytochrome oxidase activity parallels the near-infrared absorption spectrum of the oxidized form of cytochrome oxidase. More recent studies have provided evidence for the therapeutic benefit of NIR-LED treatment in the survival and functional recovery of the retina and optic nerve *in vivo* after acute injury by the mitochondrial toxin, formic acid generated in the course of methanol intoxication. The prolonged effect of brief NIR-LED treatment implies that it induces a cascade of events leading to the stimulation of gene expression, protein synthesis, and oxidative metabolism. Gene discovery studies conducted using microarray technology have provided additional insight into the mechanism of NIR-LED action in the retina. These studies have documented a significant upregulation of gene expression in pathways involved in mitochondrial energy production and antioxidant cellular protection. We also have preliminary data indicating that 670 nm LED treatment promotes retinal healing and improved visual function following high intensity laser-induced retinal injury in adult non-human primates and improves peripheral visual function in LHON patients with central vision loss. Importantly, there was no evidence of damage to the retina or optic nerve following 670 nm LED treatment in either the experimental or clinical studies. Based on these findings we propose that NIR-LED photobiomodulation represents an innovative and non-invasive therapeutic approach for the treatment of retinal injury and disease

In summary, studies by our research group in the last year of funding have demonstrated that NIR-LED treatment: (1) heals poisoned neurons by stimulating cytochrome oxidase activity; (2) protects against retinal damage and improves the recovery of retinal function in a rodent model of mitochondrial poison-induced blindness and (3) promotes retinal healing and improved visual function following high intensity laser-induced retinal injury in adult non-human primates.

VISION

Non-invasive treatment of retinal and optic nerve injury using light-emitting diode (LED) arrays delivering monochromatic light in the red to near infrared range (630-1000 nm).

BACKGROUND:

Photobiomodulation by light in the red to near infrared range (630-1000 nm) using low energy lasers or light-emitting diode (LED) arrays has been shown to accelerate wound healing, improve recovery from ischemic injury in the heart and attenuate degeneration in the injured optic nerve (19-24). At the cellular level, photoirradiation at low fluences can generate significant biological effects including cellular proliferation, collagen synthesis and the release of growth factors from cells (22, 25, 26). Our previous studies have demonstrated that LED photoirradiation at 670 nm (4 J/cm²) stimulates cellular proliferation in cultured cells and significantly improves wound healing in genetically diabetic mice (19, 24). Despite its widespread clinical application, the mechanisms responsible for the beneficial actions of photobiomodulation have not been elucidated. Mitochondrial cytochromes have been postulated as photoacceptors for red to near infrared light energy and reactive oxygen species have been advanced as potential mediators of the biological effects of this light (25, 27).

The present studies were initiated to test the hypothesis that that the therapeutic effects of red to near infrared light result, in part, from the stimulation of cellular events associated with the interaction of NIR-LED light with the photoacceptor molecule, cytochrome oxidase. **Task 1** documented the involvement of

cytochrome oxidase as a primary photoacceptor molecule for near-infrared light. **Task 2** studies examined the effects of LED photobiomodulation retinal function and retinal gene expression in rodents subjected to mitochondrial toxicity. **Task 3** studies were conducted to begin to investigate LED treatment in laser eye injury in rabbit and nonhuman primate animal models

METHODS

Materials. Light-emitting diode (GaAlAs LED) arrays (8 x 10 cm) at wavelengths of 670 nm, 728 nm, 770nm, 830 nm and 880nm were obtained from Quantum Devices, Inc. (Barneveld, WI.). Methanol (HPLC grade) obtained from Sigma Chemical Co. (St. Louis, MO) was diluted in sterile saline and administered as a 25% w/v solution. Thiobutabarbital sodium salt (Inactin) was purchased from Research Biochemicals International (Natick, MA). Atropine sulfate was obtained from AmVet Pharmaceuticals (Fort Collins, Colorado). Hydroxypropyl methylcellulose (2.5%) drops were acquired from IOLAB Pharmaceuticals (Claremont, CA). Atropine sulfate ophthalmic solution drops were purchased from Phoenix Pharmaceutical, Inc. (St. Joseph, MO). All other chemicals were reagent grade or better.

Cell Culture. To determine the optimal wavelength for activation in neurons, GaAlAs LEDs at 670nm, 728nm, 770nm, 830nm and 880nm were tested in cultured neurons, which were inactivated by TTX at a power intensity of 50 mW/cm² and energy density of 4J/cm². Studies of retinal ganglion cells and retinal pigment epithelial cells measuring DNA synthesis in response to LED treatment confirmed the effectiveness of these wavelength, power and energy parameters, thus paving the way for *in vivo* rodent retinal healing studies.

Animals. Long-Evans rats, Dutch Belted Rabbits and Cynomolgus monkeys were used in these experiments. All animals were supplied food and water *ad libitum* and maintained on a 12 hr light/dark schedule in a temperature- and humidity-controlled environment. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Methanol-Intoxication Protocol Rats were placed in a thermostatically controlled plexiglas chamber (22 x 55 x 22 cm; maintained at 22 - 23° C) and exposed to a mixture of N₂O/O₂ (1:1; flow rate 2 liters/min) for the duration of the experiment. N₂O/O₂ exposure produces a transient state of tetrahydrofolate deficiency in the rat resulting in formate accumulation following methanol administration (12). Methanol (25% w/v methanol in saline) was administered (i.p.) to N₂O/O₂ treated rats at an initial dose of 4 g/kg, followed by supplemental doses of 1.5 g/kg at 24 and 48 hours. This methanol intoxication protocol has been shown to produce a state of prolonged formic acidemia with formate concentrations between 5-8 mM in methanol-intoxicated rats resulting in visual dysfunction (6, 7). Moreover, similar concentrations of blood formate over similar time periods have been shown to produce ocular toxicity experimentally in monkeys and have been associated with visual toxicity in human methanol intoxication (8, 9, 11). Formate concentrations were determined from tail vein blood samples by fluorometric analysis (6, 7, 13).

light-emitting Diode Treatment. *In vitro:* GaAlAs LEDs at 670nm, 728 nm, 770 nm, 830nm, and 880nm were tested in cultured neurons which were inactivated by TTX at a power intensity of 50 mW/cm² and energy density of 4J/cm². *In vivo:* Rats were placed in a plexiglas restraint device (12.7 x 9 x 7.6 cm). The LED array was positioned directly over the animal at a distance of 1-inch exposing the entire body. Treatment consisted of irradiation at 670 nm for 2 min and 24 sec resulting in a power intensity of 28 mW/cm² and an energy density of 4 joules/cm² at 5, 25 and 50 hours after the initial dose of methanol. These stimulation parameters (670 nm at an energy density of 4 J/cm²) were previously demonstrated to be

beneficial for wound healing and for stimulating cellular proliferation and cytochrome oxidase activity in cultured visual neurons (19, 28).

Rodent ERG Procedures and Analyses. ERG experiments were performed as previously described (6, 7). The light stimulation apparatus consisted of a three beam optical system. All three beams were derived from tungsten-halide lamps (50W, 12V). Beam intensity was controlled by using neutral density step filters. ERG recordings were differentially amplified and computer averaged. The amplified signal was processed through a two stage active narrow bandpass filter (the half voltage of this filter was 0.2 times the center frequency). To ensure that any transients in the response that occur at the onset of the stimulus pulses were not included in the average, the initiation of signal averaging was delayed by a preset number of stimulus cycles (typically a minimum of 20). The resulting ERG is an extremely noise-free, single cycle, sinusoidal waveform. The averaged responses were measured (peak-to-trough amplitude) from a calibrated digital oscilloscope display.

Prior to ERG analysis, ophthalmoscopic examination confirmed that all eyes were free of lenticular opacities or other gross anomalies. Rats were anesthetized with thiobutabarbital sodium salt (100 mg/kg, i.p.), positioned in a Kopf stereotaxic apparatus and placed on a heating pad to maintain core body temperature at 37° C. Atropine sulfate (0.05 mg/kg, s.q.) was administered to inhibit respiratory-tract secretions. The pupil of the eye to be tested was dilated by topical application of 1% atropine sulfate. Methylcellulose was topically applied as a lubricant and to enhance electrical conduction. A circular silver, wire recording electrode was positioned on the cornea, a reference electrode was placed above the eye, and a ground electrode was placed on the tongue. Recordings were obtained under ambient light conditions from cool white fluorescent room lights approximately 100 cd/m² at the rat's eye. Flickering stimuli (light: dark ratio = 0.25: 0.75) were presented. Responses to 60 successive flashes were averaged for each stimulus condition. At each test wavelength, a minimum of four stimulus intensities spaced at intervals of 0.3 log unit, were presented. The stimulus intensity yielding a 5μV criterion response was determined by extrapolating between the two intensity points that bracketed the 5μV response for each animal. All sensitivity measures were made in triplicate.

Two experimental protocols were employed to evaluate retinal function. (1) *15 Hz/510 nm ERG Response:* ERGs were recorded in response to a 15 Hz flickering light at a wavelength of 510 nm over a 3 log unit range of light intensity. For these studies, the unattenuated stimulus (log relative retinal illumination = 0) had an irradiance of 25 μW distributed over the 70° patch of illuminated retina. This can be calculated to produce retinal illumination equivalent to about 10⁴ scotopic trolands. These recording conditions disadvantage rods; however, since at least 97% of rat photoreceptors are rods and ERGs are recorded at luminance intensities ranging from 10¹ - 10⁴ scotopic trolands, it is likely that the responses to the 15 Hz/510 nm light are drawn from both rods and medium wavelength cones (M-cones) (6, 7, 32). (2) *25Hz/UV ERG Response:* UV-sensitive cone responses were elicited by a 25 Hz flickering ultraviolet light (380 nm cut off) in the presence of an intense chromatic adapting light (445 μW) which eliminated responses mediated by rods and M-cones (32). 25Hz/UV ERG responses were recorded over a 1.5 log unit range of light intensity. For these studies, the unattenuated stimulus (log relative retinal illumination = 0) had an irradiance of 25 μW distributed over the 70° patch of illuminated retina. This can be calculated to produce retinal illumination equivalent to about 10⁴ scotopic trolands in the rat eye.

Rabbit and Primate Visual Function Assessments Prior to testing, each animal received a complete ophthalmological examination, including visualization of the fundus using indirect ophthalmoscopy, intraocular pressure (IOP) measurement, and examination of the anterior chamber by slit-lamp biomicroscopy. Stereo color photographs were obtained from each eye using a digital fundus camera. The integrity and thickness of the retinal layers in the macular region also were assessed by optical coherence tomography (OCT). The functional integrity of the macula was assessed by mERGs. Two mERG stimuli were used: 1) 103 equal sized hexagonal elements each subtending 4.4 deg; and 2) 241 equal sized hexagonal elements each subtending 2.2 deg.

Laser-induced Injury: Following initial screening and baseline testing, a grid of laser burns was applied to the macula by Dr. T. M. Nork, M. D., a board-certified retinal surgeon. The individual spots were approximately 75-150 microns in diameter, 75-120 mW in strength and 0.05-0.2 sec in duration. The grid extended to about 10 arc degrees from the foveal center in all directions (20° diameter).

NIR-LED treatment: One hour following macular grid laser and mERG assessment, LED treatment was delivered. The treatment consisted of 670 nm light delivered for 1 min 45 sec, at an intensity of 38 mW/cm², resulting in an energy density of 4 J/cm² at the target tissue. LED treatments will be repeated at 24, 48, 72 and 96 h following creation of macular laser wounds. Functional testing (mERG and VEP) of both eyes will be repeated at 72 h and 100 h following creation of macular laser wounds. At 100 h the stereo color retinal photographs and OCT images will be obtained from each eye.

Retinal and Visual Function Analysis: The mERG results in a ‘first-order kernel’ voltage v. time waveform that consists of a series of negative and positive waves. Each wave has an associated amplitude and implicit time (delay). The mERG software allows the individual mERG waves to be averaged into groups suitable for the design of a particular experiment. The waves corresponding to the macular region where the laser wound was produced were averaged. The amplitude and implicit time of the first four waves (n1, p1, n2, p2) of the first-order kernel were submitted to an analysis of variance (ANOVA) with Time (baseline, 1 h post, 100 h post), Eye (laser, fellow), and Group (treated, untreated) as factors. Appropriate repeated measures statistical procedures (Greenhouse-Geiser corrections) were used. In addition, means, error estimates percent recovery statistics were graphically presented.

Histopathologic analysis. Retinal tissue was prepared for light and electron microscopic analysis as described by Seme et al. (2001). The retina, LGN and visual cortex of laser-injured and NIR-LED-treated animals was processed for cytochrome oxidase histochemistry at the Medical College of Wisconsin to determine potential benefits of LED-therapy on retinal and cortical neurons.

DNA microarray gene discovery studies: Specific genes that show altered expression were identified using DNA microarray technology at WRAIR Molecular Pathology Laboratory using either commercial gene arrays or glass slides or using our custom human chips for monkey samples. For glass slides fluorescent labeling was performed and scanned in the AXON scanner. The data was analyzed with Gene Pix software. Statistical, clustering, sorting parameters were applied by using Gene Spring and Partek software.).

Statistical Analysis. All values are expressed as means \pm SEM. A one-way ANOVA with Bonferroni’s test was used to determine whether any significant differences existed among groups for blood formate concentrations. For ERG studies, a two-way ANOVA was performed. In all cases, the minimum level of significance was taken as $P < 0.05$.

RESULTS

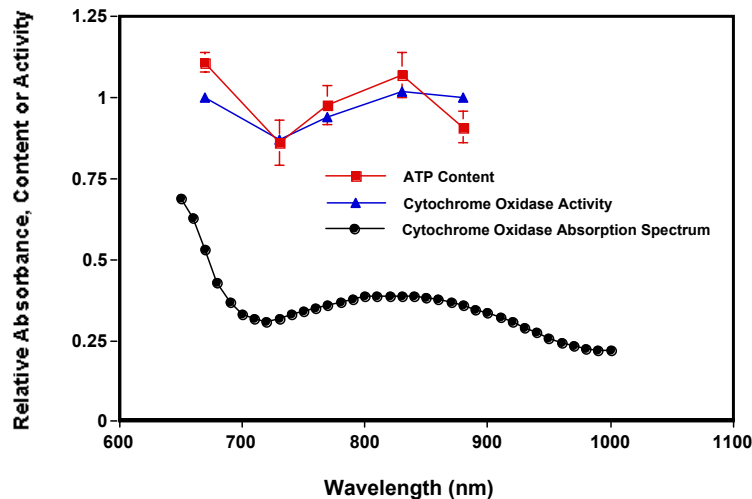
Task 1: To document the involvement of cytochrome oxidase as a primary photoacceptor molecule for near-infrared light.

Hypothesis: We hypothesize that the therapeutic effects of red to near infrared light result, in part, from the stimulation of cellular events associated with increases in cytochrome c oxidase activity.

Milestone: LED light at wavelengths corresponding to absorption peaks of copper centers in the cytochrome oxidase molecule are most effective in stimulating cytochrome oxidase activity and stimulating ATP synthesis.

Results and Discussion: In NIR-LED therapy there are four important treatment variables that must be addressed to optimize any therapeutic regimen. (1) Energy density or fluence, (2) NIR-LED wavelength (3) number of treatments and (4) treatment interval. With respect to energy density, we are confident that treatment at an energy density of 4 J/cm^2 is within the optimal range. This confidence is based on a large body of evidence from our studies and those of other investigators which documents that exposure to near-infrared light at energy densities (fluence) between $2 - 10 \text{ J/cm}^2$ promotes mitochondrial energy metabolism, cell division, wound healing, protects against retinal damage and improves the recovery of retinal function in following retinal damage by a mitochondrial poison and promotes retinal healing and improved visual function following high intensity laser-induced retinal injury. With respect to LED wavelength, the majority of our studies have been conducted using 670 nm LED light and we have substantial evidence that NIR-LED treatment at 670 nm is beneficial both *in vitro* and *in vivo*. In studies investigating additional NIR wavelengths, we have shown that the recovery of neuronal cytochrome oxidase activity and cellular ATP content correlates with the cytochrome oxidase absorption spectrum (**Figure 1**). These studies showed that LED light at wavelengths corresponding to the absorption peaks of the copper centers in the cytochrome oxidase molecule (670 nm, 830nm and 880 nm) were most effective in promoting the recovery of cytochrome oxidase activity in cultured primary neurons.

Figure 1: Recovery of Neuronal Cytochrome Oxidase Activity and Cellular ATP Content Correlates with Cytochrome Oxidase Action Spectrum)



We also have evidence that multiple treatments are more effective than a single treatment in stimulating cytochrome oxidase activity and the three NIR-LED treatments administered at 5, 25, 50 hours following toxin exposure protects against and reverses toxin-induced retinal toxicity in rat. In the monkey, 5 treatments administered 1, 24, 48, 72 and 96 hours post-injury were effective in ameliorating high-energy laser-induced retinal injury.

Deliverable to DARPA: *In vitro* evidence that the cytoprotective effects of red to near infrared light result, in part, from the stimulation of cellular events associated with increases in cytochrome oxidase activity.

Task 2: To examine the effects of LED photobiomodulation retinal function and retinal gene expression in an animal model of toxin induced retinal mitochondrial dysfunction.

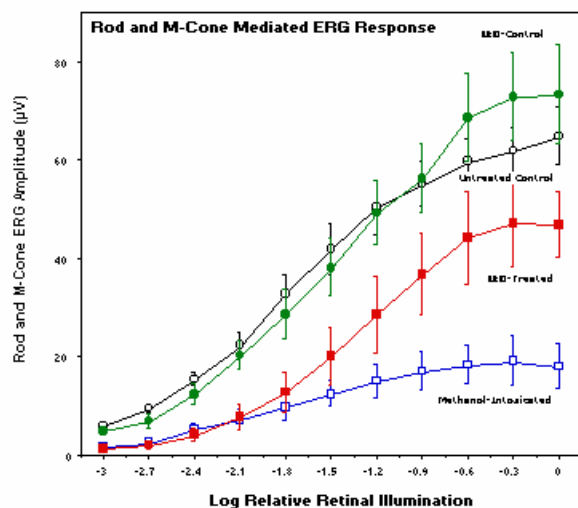
Hypothesis: The prolonged effects of brief LED treatment result from the induction of a cascade of events leading to the stimulation of gene expression, protein synthesis, and oxidative metabolism.

Milestones:

1. Three brief NIR LED treatments produced rapid recovery of retinal function following toxic retinal injury.
2. NIR LED-treatment induced the expression of genes which code for proteins involved in oxidative metabolism and cellular protection. (collaboration with Dr. Marti Jett at WRAIR, Wash. D.C.)

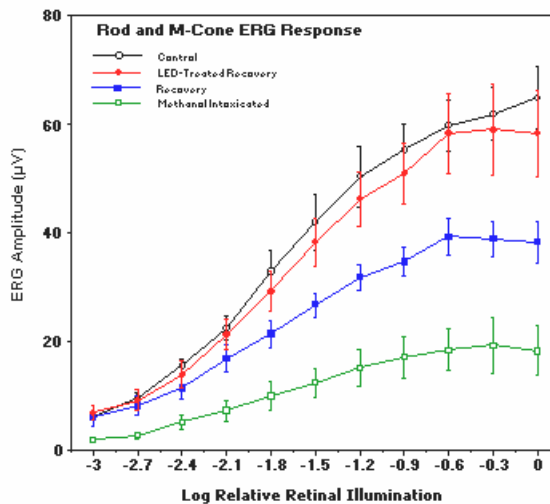
Results and Conclusions: *Retinal Function Studies:* Methanol intoxication produces toxic injury to the retina and optic nerve resulting in blindness. The toxic metabolite in methanol intoxication is formic acid, a mitochondrial toxin known to inhibit the essential mitochondrial enzyme, cytochrome oxidase. Studies were undertaken to test the hypothesis that exposure to monochromatic red radiation from light-emitting diode (LED) arrays would protect the retina against the toxic actions of methanol-derived formic acid in a rodent model of methanol toxicity. Using the electroretinogram as a sensitive indicator of retinal function, we demonstrated that 3 brief (2 min. 24 sec.) 670 nm LED treatments (4 J/cm²), delivered at 5, 25 and 50 hours of methanol intoxication, significantly attenuated the retinotoxic effects of methanol-derived formate during intoxication (**Figure 2**) and profoundly improved the recovery of retinal function following intoxication (**Figure 3**). We further show that LED treatment protected the retina from the histopathologic changes induced by methanol-derived formate (ref or figure). These findings provide a link between the actions of monochromatic red to near infrared light on mitochondrial oxidative metabolism *in vitro* and retinoprotection *in vivo*. They provide the basis for phase II studies directed at examining laser injury in a rodent model

Figure 2



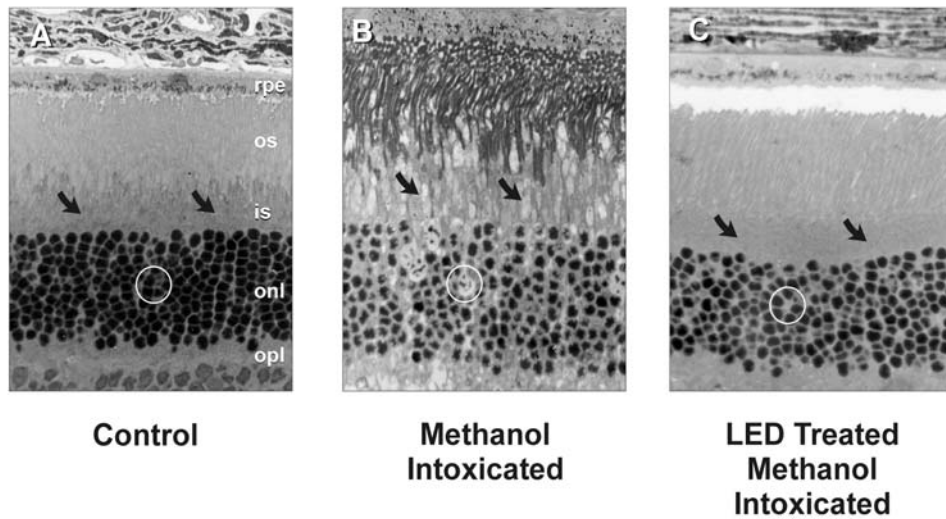
670 nm NIR-LED Protects Against Retinal Toxicity

Figure 3



670 nm NIR-LED Improves Recovery

Figure 4: Histological Evidence of NIR-LED protection of the retina.



Gene Expression Studies: We have compared gene expression profiles in the neural retina of untreated rats with those from the neural retina of methanol-intoxicated rats and LED-treated methanol-intoxicated rats. Results from these studies indicate that methanol intoxication and LED treatment altered the retinal expression of nearly 80 genes. At least 26 of these genes that were up-regulated in the retinas of methanol intoxicated rats were correspondingly down-regulated by in the retinas of LED treated methanol intoxicated rats and vice-versa (**Figure 5**). Several functional subcategories of genes regulated by NIR-LED were identified in retinal samples, including those encoding DNA repair proteins, antioxidant defense enzymes, molecular chaperones, protein biosynthesis enzymes, and trafficking and degradation proteins. Striking differences were observed in genes from cytochrome oxidase family, peroxiredoxin family and genes involved in cell growth and maintenance (**Figure 6**). Differential expression of selected genes was confirmed at the level of RNA. We intend to further substantiate these findings using real time PCR, Northern and Western analysis and to investigate the roles of several of these genes in cellular energy production and cellular survival

Retinal Genes Up or Down Regulated by Methanol Intoxication +/- LED Treatment

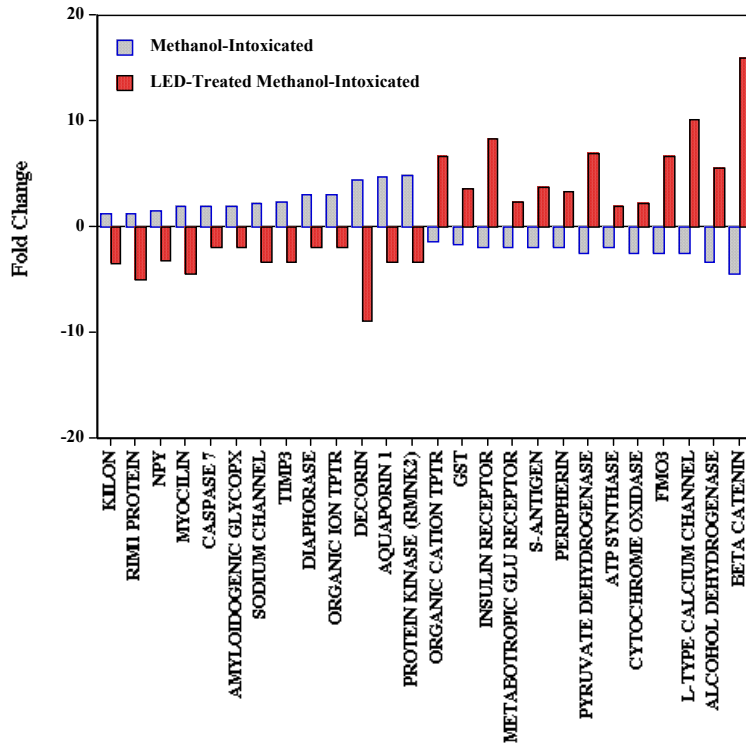


Figure 5: Up and Down Regulation of Genes in the retinas of methanol-intoxicated vs NIR-LED-treated methanol-intoxicated rats.

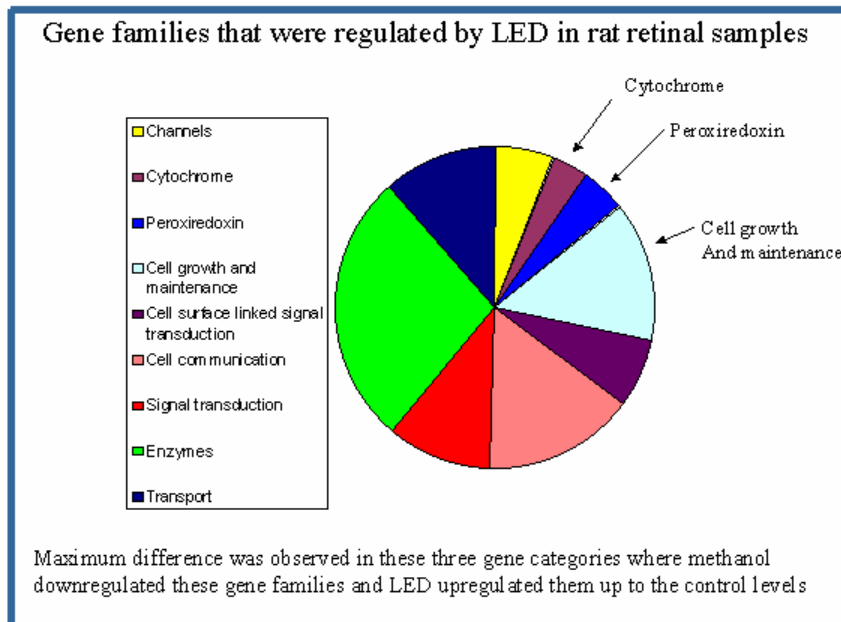


Figure 6: Gene families regulated by NIR-LED treatment in rat retina

In summary, our studies to date have revealed a great many potential markers and have provided some explanations as to the mechanisms by which LED exposure may enhance the wound healing process. The next phase of this project will build on these initial efforts and focus on the military relevant problem of retinal repair. The team assembled by Dr. Whelan includes highly experienced civilian and military ophthalmologists who will measure various parameters in order to quantitatively determine functional retinal repair +/- LED treatments. Using our sophisticated molecular techniques as impartial markers, we will further characterize the process and optimize healing of retinal injuries to understand the chain of events that lead to improved wound healing.

Deliverable to DARPA: Evidence that brief LED treatment up-regulates the expression of genes important in cellular survival and down-regulates the expression of genes involved in cell death pathways.

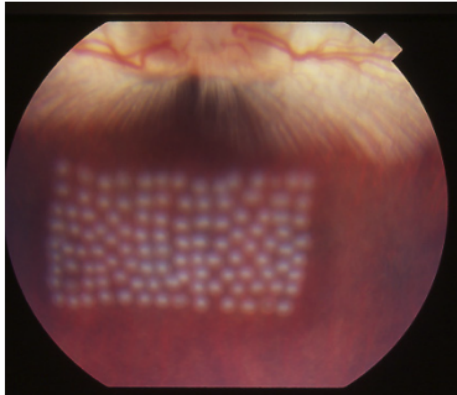
Task 3 To investigate NIR-LED treatment in laser eye injury in rabbit and non-human primate animal models.

Hypothesis: NIR-LED treatment will improve retinal healing and visual function following high intensity laser injury.

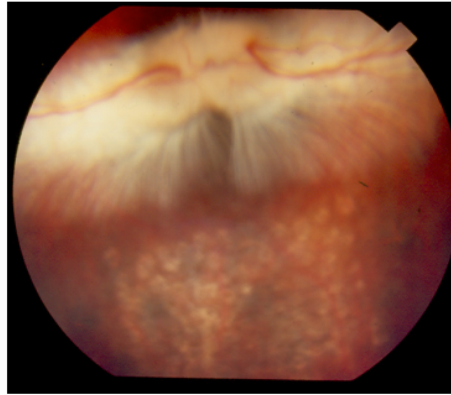
Rationale/Approach: These studies were conducted by the team at the University of Wisconsin, Madison (J.Ver Hoeve) in collaboration with the MCW group on cynomolgus monkeys (*Macaca fascicularis*). Following baseline assessments (two baseline mERGs/VEPS) (multifocal ERGs/visual evoked potentials), all monkeys will receive laser wounds to the macula of one eye. The fellow eye will not be lasered. Treated monkeys will receive LED treatment to the lasered eye and the fellow eye. LED treatment will consist of 1 min 45 sec of 670 nm light at 4 J/cm² delivered at 1, 24, 72, and 96 h following the laser wound. Other LED parameters will also be used, as dictated by the results of Tasks 1 & 2. Additional monkeys will serve as controls (no LED treatment). The functional effect of LED treatment will be assessed by comparing mERG and VEP parameters in the treated eyes with the untreated eyes of the control monkeys immediately following the laser wound and at 72 h and at 100 h, prior to sacrifice. In addition, the possibility of deleterious functional effects of the LED treatment will be assessed by comparing the fellow (non-lasered) eyes with baseline values and with the non-lasered eyes of the control monkeys. For logistical reasons, monkeys will be tested in pairs (treated and control).

Preliminary Data in the Rabbit Model: To optimize LED treatment parameters *in vivo*, we have examined laser retinal injury in a rabbit animal model. To date we have performed one experiment using this animal model. Two rabbits were used in this study- one lasered without LED treatment and one lasered with LED (treatment at 24,48 and 72 h post injury. A laser grid was created in the central retina of right eye of each animal (**Figure 7**). The LED treated rabbit fundus had fewer distinct laser spots 1-week following laser injury (**Figure 7**). These preliminary findings are indicative of improved retinal healing following LED treatment in laser injured rabbit model.

Figure 7: High energy laser grid shows evidence of improved healing in eye treated with NIR-LED



Rabbit retinal LASER injuries without LED



Rabbit retinal LASER injuries with LED therapy

Preliminary Data in Nonhuman Primates: We have initiated studies of laser retinal injury in a nonhuman primate model. To date, we have performed two experiments using this animal model. In each experiment one monkey was lased without LED treatment and one lased with LED treatment (670 nm, 4 J/cm²). A laser grid (128 spots delivered to the macula and perimacula) was created in the central retina of right eye of each animal (**Figure 8**) This grid consisted of grade I and II burns, photocoagulating the photoreceptors and outer nuclear layer of the retina. Multifocal ERG was performed to assess the functional state of the retina. In the first experiment, the LED-treated monkey was treated at 1, 24, 72 and 96 h post injury. ERG amplitude in both LED treated and untreated monkeys was temporarily increased shortly after laser injury and this increase was greater in the LED-treated monkey. Assessment of the severity of the laser burn in LED treated and untreated animal demonstrated a greater than 50% improvement in the degree of retinal healing at 1 month post-laser in the LED-treated monkey (**Figure 9**). In addition, the thickness of the retina measured at the fovea by optical coherence tomography did not differ from the pre-laser thickness in the LED-treated animal whereas it was 50% thinner in the untreated animal (**Figure 8**). Importantly, LED treatment prevented the loss of cytochrome oxidase staining in the lateral geniculate nucleus (**Figure 10**) clearly showing that the brain was responding to visual input from the “healed” retina in the LED-treated animal much more effectively than in the untreated animal.

In the second study, the LED-treated animal was treated once per day for 11 days and mfERG recordings were recorded. Again, shortly after laser injury, the ERG amplitude was temporarily increased in both LED treated and untreated animals. However, in this experiment the increases were comparable. (**Figure 11**) At 4 days post laser injury, the mfERG responses in LED treated and untreated animals had decreased to pre-laser amplitudes. However, by day 11 post laser, the mfERG response in the LED treated monkey was more than 50% greater than that measured in the untreated (sham) monkey. In both experiments, these preliminary findings are indicative of improved retinal healing and visual cortical function following LED treatment in laser injured primate model. It must be stressed that these findings are preliminary and it essential that this study be replicated and expand upon.

Figure 8: Laser grid appearance and foveal thickness in control (left) and NIR-LED (right) treated monkey retina

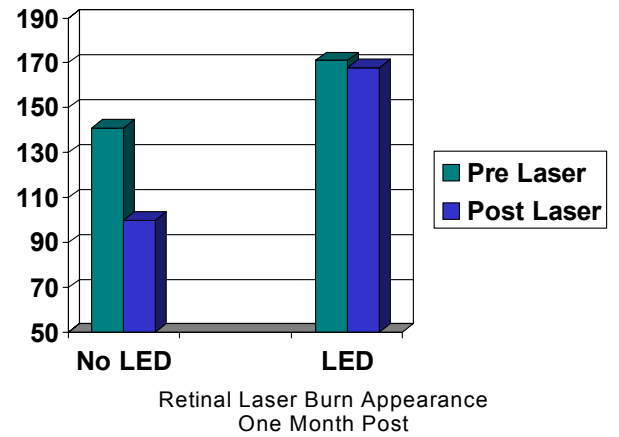
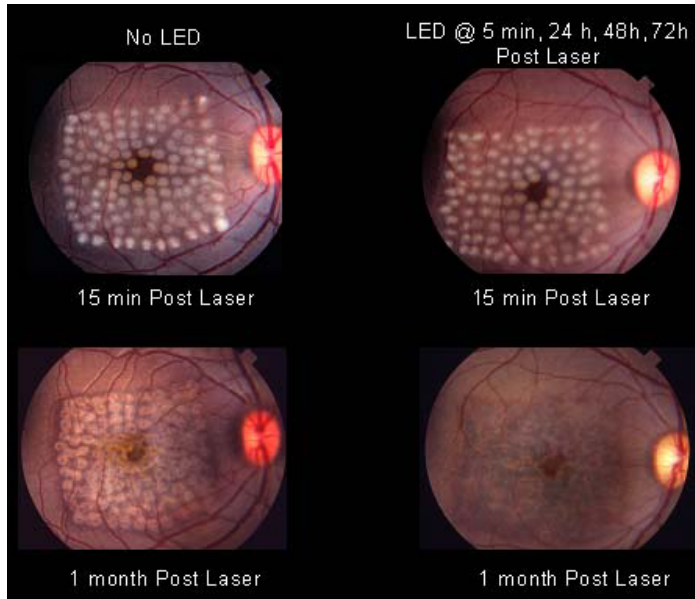


Figure 9: NIR-LED Treatment Stimulates Retinal Healing following laser injury.

Grading Scale: Higher values are indicative of less complete healing. 0 = spot faded, no distinct borders; 0.5 = portion of Border visible; 1.0 = borders of entire spot visible. (t-test)

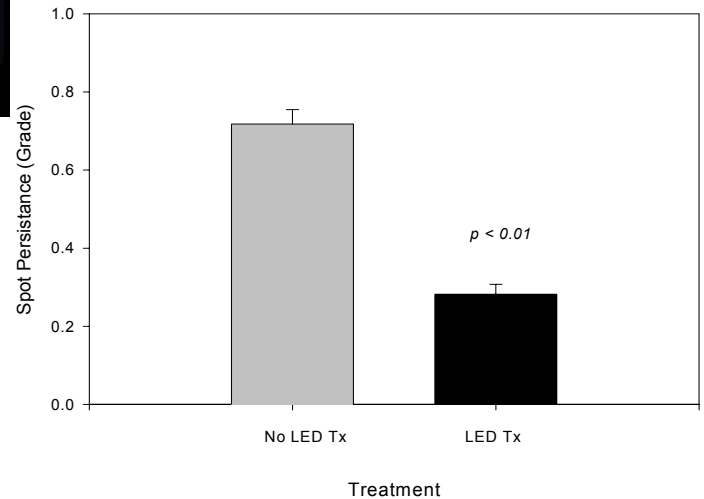
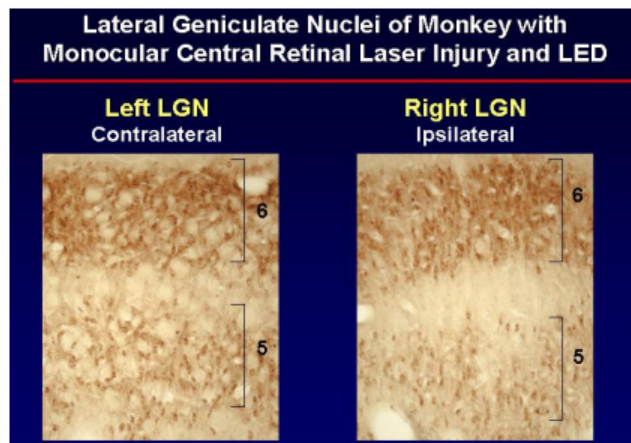
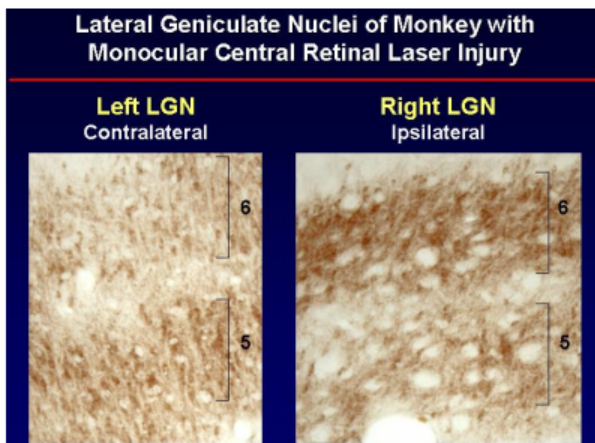
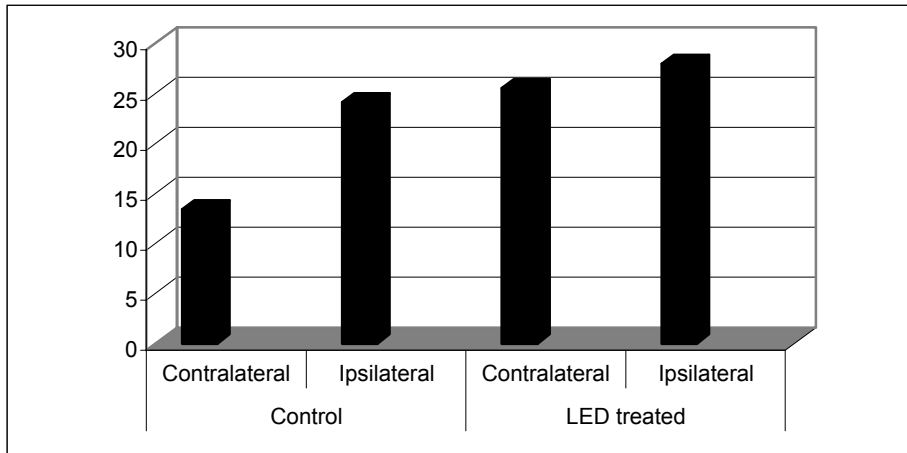


Figure 10: Quantitative Analysis of Cytochrome oxidase histochemistry in LGN of control laser-treated monkey and NIR-LED treated laser treated monkey



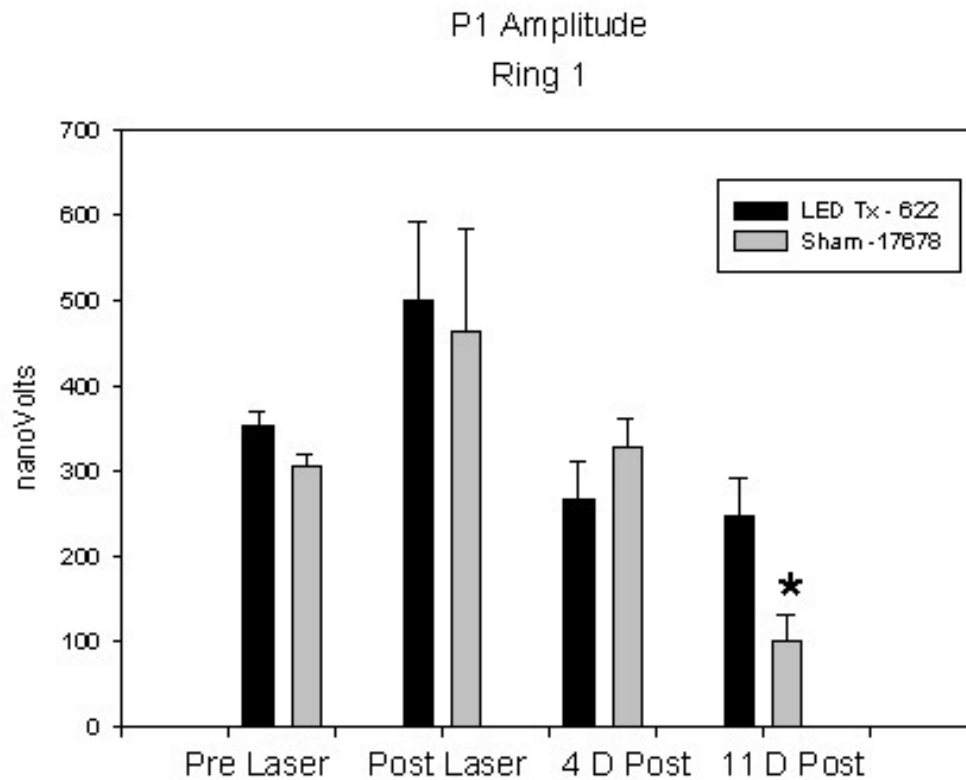


Figure 11: NIR-LED treatment significantly improves retinal function in a nonhuman primate model of laser eye injury

Deliverable to DARPA: Evidence that Photobiomodulation may provide an innovative and non-invasive therapeutic approach for the treatment of retinal and optic nerve injury.

DISCUSSION

Low energy photon irradiation by light in the far red to near infrared spectral range (630-1000 nm) using low energy lasers or light emitting diode arrays has been found to modulate various biological processes in cell culture and animal models (22-25). This phenomenon of photobiomodulation has been applied clinically in the treatment soft tissue injuries and to accelerate wound healing (19, 22). The mechanism of photobiomodulation by red to near infrared light at the cellular level has been ascribed to the activation of mitochondrial respiratory chain components resulting in initiation of a signaling cascade which promotes cellular proliferation and cytoprotection (25, 27, 28). A comparison of the action spectrum for cellular proliferation following photoirradiation with the absorption spectrum of potential photoacceptors lead Karu and colleagues to suggest that cytochrome oxidase is a primary photoreceptor of light in the red to near infrared region of the spectrum (25). We have confirmed and extended these observations showing that the NIR absorption spectrum of cytochrome oxidase correspond with the action spectrum for upregulation of cytochrome oxidase activity and cellular ATP content.

Studies conducted in primary neuronal cultures by our research group have shown that 670 nm LED photobiomodulation reversed the reduction in cytochrome oxidase activity produced by the blockade of voltage-dependent sodium channel function by tetrodotoxin and up-regulated cytochrome oxidase activity in normal neurons (28). Additional studies have extended these investigations to an *in vivo* system to determine if 670 nm LED photobiomodulation would improve retinal function in an animal model of formate-induced mitochondrial dysfunction. Results of this study demonstrate the therapeutic benefit of photobiomodulation in the survival and functional recovery of the retina *in vivo* after acute injury by the mitochondrial toxin, formic acid generated in the course of methanol intoxication. We provide *in vivo* evidence that 3 brief post-methanol-intoxication treatments with 670 nm LED photoirradiation promotes the recovery of retinal function in rod and cone pathways and protects the retina from the histopathologic changes induced by methanol-derived formate. These findings provide a link between the actions of red to near infrared light on mitochondrial oxidative metabolism *in vitro* and retinoprotection *in vivo*.

Low energy laser irradiation has documented benefits in promoting the healing of hypoxic, ischemic, and infected wounds (19, 22). However, lasers have limitations in beam width, wavelength capabilities, and size of wounds that can be treated (19). Heat generated from the laser light can damage biological tissue, and the concentrated beam of laser light may accidentally damage the eye. Light-emitting diode (LED) arrays were developed for NASA manned space flight experiments. In comparison to lasers, the patented LED technology generates negligible amounts of heat, is clinically proven to be safe, and has achieved non-significant risk status for human trials by the FDA (19). The wavelength, power, and energy parameters used in the present study are based on their beneficial effects for wound healing in human's (19) and stimulation of CO activity in cultured neuronal cells (28).

The prolonged effect of 3 brief LED treatments in mediating the cytoprotective actions in cultured retinal cells and the retinoprotective actions in methanol intoxication suggests that 670 nm LED photostimulation induces a cascade of signaling events initiated by the initial absorption of light by cytochrome oxidase. These signaling events may include the activation of immediate early genes, transcription factors, cytochrome oxidase subunit gene expression and a host of other enzymes and pathways related to increased oxidative metabolism (25, 28, 44). In addition to increased oxidative metabolism, red to near infrared light stimulation of mitochondrial electron transfer is also known to increase the generation of reactive oxygen species (25). These mitochondrially generated reactive oxygen species may function as signaling molecules to provide communication between mitochondria and the cytosol and nucleus and thus play an important signaling role in the activation of retinoprotective processes following LED treatment (45).

The results of this study and others suggest that photobiomodulation with red to near infrared light augments recovery pathways promoting neuronal viability and restoring neuronal function following injury. Importantly, there was no evidence of damage to the normal retina following 670 nm LED treatment. Based on these findings, we suggest that photobiomodulation may represent an innovative and novel therapeutic approach for the treatment of retinal injury as well as the treatment of retinal diseases, including age-related macular degeneration, glaucoma, diabetic retinopathy, and Leber's hereditary optic neuropathy.

ACKNOWLEDGEMENTS

We thank Kurt Henry, M.D. for helpful discussions and suggestions and Anna Fekete for excellent technical assistance. We also gratefully acknowledge the technical support of Quantum Devices, Inc. for providing the

light emitting diode arrays for this project. This work was supported by Defense Advanced Research Projects Agency Grants DARPA N66001-01-1-8969 & N66001-03-8906 (to H.T.W.), National Institute of Environmental Health Sciences Grant ES06648 (to J.T.E), National Eye Institute Core Grant P30-EY01931 (J.T.E. and M.W.-R. Core Investigators), National Eye Institute Grants EY11396 (J.T.E) and EY05439 (M.W.-R.), and the Bleser Foundation Endowed Professorship (H.T.W.).

REFERENCES

1. Rizzo J. F. (1995) *Neurology*. **45**, 11-16.
2. Baron, M.J., Johnson, M.A., Andrews, R.M., Clarke, M.P., Griffiths, P.G. Bristow, E., He, L.P., Durham, S. & Turnbull, D. M. (2001) *Invest. Ophthalmol. Vis. Sci.* **42**, 3016-3022.
3. Carelli, V., Ross-Cisneros, F.N. & Sadun A.A. (2002) *Neurochem. Int.* **40**, 573-584.
4. Hayreh M. M., Hayreh S.S., Baumbach G.L, Cancilla P, Martin-Amat G., & Tephly T.R. (1980) in. *Neurotoxicity of the Visual System*. Eds Merigan W. & Weiss B. (Raven Press, New York) pp. 35-53.
5. Martin-Amat, G., Tephly T.R., K. E. McMartin K.E., Makar, A.B., Hayreh, M. M., S. S. Hayreh, S.S., Baumbach, G., & Cancilla, P. (1977). *Arch. Ophthalmol.* **95**, 1847-1850.
6. Seme, M.T., Summerfelt, P. M., Henry, M.M, Neitz, J. & Eells, J.T. (1999) *J. Pharmacol. Exp. Ther.* **289**, 361-370,
7. Seme M.T., Summerfelt P., Henry M.M., Neitz J. & Eells J.T. (2001) *Invest. Ophthalmol. Vis. Sci.* **42**, 834-841.
8. Eells J.T. (1992) in *Browning's Toxicity and Metabolism of Industrial Solvents. Vol. IV Alcohols and Esters*. Eds Thurman, R.G. & Kaufmann, F.C. (Elsevier, Amsterdam) pp. 3-15.
9. Kavet R. & Nauss K. (1990). *Crit Rev Tox* **21**: 21-50.
10. Sharpe J., Hostovsky M., Bilbao J. & Rewcastle N.B. (1982) *Neurology*. **32**, 1093-1100.
11. Ingemansson S.O. (1983). *Acta Ophthalmol Suppl* **158**, 5-12.
12. Eells J.T., Henry M.M., Lewandowski M.F., Seme M.T & Murray T.G. (2000) *Neurotox* . **21**, 321-330.
13. Murray T.G., Burton T.C., Rajani C, Lewandowski M.F., Burke J.M., & Eells J.T. (1991) *Arch Ophthalmol.* **109**,1012-1016.
14. Lee E.W., Garner C.D. & Terzo T.S. (1994) *Toxicol. Appl. Pharmacol.* **128**: 199-206.
15. Martin-Amat G., McMartin K.E., Hayreh S.S., Hayreh M., & Tephly T.R. (1978) *Toxicol Appl Pharmacol.* **45**, 201-208
16. Nicholls P. (1975) *Biochem Biophys Res Commun.* **67**: 610-616.
17. Nicholls P. (1976). *Biochim Biophys Acta.* **430**:13-29.
18. Wong Riley, M.T.T. (1989) *Trends Neurosci.* **12**, 94-101.
19. Whelan, H.T., Smits, R.L., Buchmann, E.V., Whelan, N.T., Turner, S.G., Margolis, D.A., Cevenini, V., Stinson, H., Ignatius R., Martin T. et al. (2001). *J. Clin. Laser Med. Surg.* **19**, 305-314.

20. Oron, U., Yaakobi, T., Oron, A., Hayam, G., Gepstein, L., Rubin, O., Wolf, T. & Ben Haim, S. (2001) *Lasers Surg. Med.* **28**, 204-211.
21. Assa, E.M., Rosner, M., Belkin, M., Solomon, A. & Schwartz, M. (1989) *Brain. Res.* **476**, 205-212.
22. Conlan, M.J., Rapley, J.W., & Cobb, C.M. (1996). *J. Clin. Periodont.* **23**, 492-496.
23. Yu W, Naim J.O., Lanzafame R.J., (1997) *Lasers Surg. Med.* **20**, 56-63.
24. Sommer A.P., Pinheiro A.L., Mester A.R., Franke R.P., & Whelan H.T. (2001) *J. Clin. Laser Med. Surg.* **19**, 27-33.
25. Karu T. (1999). *J. Photochem. Photobiol.* **49**, 1-17.
26. Leung, M.C.P., Lo, S.C.L., Siu, F.K.W. & So, K.F. (2002) *Lasers Surg. Med.* **31**, 283-288.
27. Grossman, N. Schneid, N., Reuveni, H., & Lubart, R. (1998) *Lasers Surg. Med.* **22**, 212-218
28. Wong-Riley M.T.T., Bai X., Buchmann E., & Whelan H.T. (2001) *NeuroReport.* **12**, 3033-3037
29. Koskelainen A, Donner K, Kalamkarov G & Hemila S (1994) *Vision Res* **34**, 983-994.
30. Findl O, Hansen R and Fulton A (1995) *Invest Ophthalmol Vis Sci* **36**, 1019-1026.
31. Fox D.A. & Katz, L. (1992) *Vision Res* **32**, 249-255.
32. Jacobs G, Neitz J, Deegan J. (1991) *Nature.* **353**, 655-656.
33. Ames III, A., Y. Li, Y., E.C. Heher, E.C. & Kimble, C.R. (1992) *J. Neurosci.* **12**, 840-853.
34. Ames III, A. (1992) *Can. J. Physiol. Pharmacol.* **70**, S158-S164.
35. McKelvie P.A., Morley J.B., Byrne E. & Marzuki, S. (1991) *J. Neurol. Sci.* **102**, 51-60.
36. Rapp L.M., Tolman BL. & Dhindsa H.S. (1990) *Invest. Ophthalmol. Vis. Sci.* **31**, 1186-1190.
37. Runge P., Calver, D., Marshall, J., & Taylor, D. (1986) *Br J Ophthalmol* **70**, 782-796.
38. Yu, W., Naim, J.O., McGowan, M. Ippolito, K., & Lanzafame, R.J. (1997) *J. Photochem. Photobiol.* **66**, 866-871.
39. Passarella, S., Casamassima, E., Molinari, S, Pastore, D., Quagliariello, E., Catalino, I.M. & Cingolani, A. (1984) *FEBS Lett.* **175**, 95-99.
40. Pastore, D., Greco, M. & Passarella, S. (2000) *Int. J. Radiat. Biol.* **76**, 863-870.
41. Cooper, C.E. & Springett, R. (1977) *Phil. Trans. R Soc. Lond. B. Biol. Sci.* **352**, 9-676.
42. Beauvoit, B., Evans, S.M., Jenkins, T.W., Miller, E.E., & Chance B. (1995). *Anal. Biochem.* **226**, 167-174.
43. Beauvoit, B., Kitai, T., & Chance B. (1994). *Biophys J.* **67**, 2501-2510.
44. Zhang C. & Wong-Riley, M. (2000) *Eur. J. Neurosci.* **12**, 1013-1023
45. Nemoto, S., Takeda, K., Yu, Z.X., Ferrans, V.J. & Finkel, T. (2000) *Mol. Cell. Biol.* **20**, 7311-7318.

