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TITLE: Elucidation of Inflammation Processes Exacerbating Neuronal Cell Damage to the Retina and Brain Visual Centers as Quest for Therapeutic Drug Targets in Rat Model of Blast Overpressure Wave Exposure.

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14. ABSTRACT A frequent cause of traumatic eye injuries to soldiers is exposure to blast shock waves; and it can involve cellular damage to the retina as well as brain visual centers. Since there are relatively few animal studies that have studied this, there is an urgent need to advance the characterization of blast induced visual system injuries and identify potential drug therapies. Inflammation plays a key role in the destruction of injured neuronal tissues, as carried out by immune cells; and thus is a promising target. Scope and timing, however, of this process must be better understood. Our study uses an adult rat model of eye and brain injuries, as produced by exposure to simulated blast waves in a shock tube. Rats were kept on an omega-3 polyunsaturated fatty acid deficient diet, which promotes inflammation. Conversely, some are fed an omega-3 enriched diet by ocean fish oil supplementation. Up to one month after blast, eye (retina) and brain damage was assessed by electroretinography (ERG), visual acuity task, magnetic resonance imaging (MRI), histopathology, and immunoassay arrays for inflammation signaling factors. Our findings revealed that blast exposure leads to long lasting impairments of visual function (i.e., out to 28 days) with an acute underlying infiltration of activated immune cells (e.g. macrophages) in the retina and brain (i.e., by 3 days), which is accompanied by elevated cytokines and degeneration of neurons. Recovery of retinal function is very gradual with reoccurring signs of neuroinflammation. Despite having potent anti-inflammatory properties, continuous high doses of dietary omega-3 showed only a modest ability to alleviate these injury events, which was most apparent at the chronic phase. Thus, omega-3 supplementation is a supportive rather than first line treatment approach. Overall, our mission is to provide results that will lead to new therapeutic countermeasures for blast-induced damage sustained to the visual system of US Army personnel.					
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I. INTRODUCTION:

The objective of the project is to identify mitigation and treatment strategies for blast-related traumatic injuries to the neuronal components of the visual system, as frequently suffered by US Military personnel in current fields of operation. These can involve grave damage to the eyes (retina) as well as brain visual processing centers. Despite the difficult long term disability that loss of vision represents, there are relatively few animal studies that have characterized blast-induced neuronal injuries to the visual system, and searched for promising drug based therapies. In rats exposed to blast over pressure waves, we have already documented marked retinal signaling dysfunction caused by photoreceptor degeneration, which extends to central brain pathways associated with vision (e.g., optic tract, lateral geniculate nucleus, and superior colliculus). Based on our own preliminary data and that published by others showing accumulation of activated microglia and macrophages in the retina and brain following blast, we hypothesize that immune cell mediated processes play a primary role dictating the extent of blast-induced neurodegeneration in the visual system. Thus, our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in retina and brains of adult male rats after blast wave exposure, using a compressed air driven shock tube, so as to discern potential drug targets and therapeutic windows. To effectively utilize this model to identify therapies, it is crucial to understand the biochemistry underlying the injuries' progression. In particular, scope and timing of immune cell infiltration and cytokine release requires rigorous characterization. Nutritional impact on blast injury vulnerability also will be studied under pro-inflammatory conditions, i.e., a diet deficient versus one enriched in omega-3 polyunsaturated fatty acids, including both docosahexaenoic and eicosapentaenoic acids (DHA and EPA, respectively), as provide by daily supplementation with high dose ocean fish oil. This will enhance our ability to monitor immune cell processes in neuronal structures for discovery of unique drug targets. We will assess the health status of the rat's retina and brain following the blast insult at acute and chronic time points, i.e., 3, 7, 14, and 28 days. Outcome measures that will be used are electroretinography (ERG) for neuronal signaling to a light stimulus; visual acuity task (optokinetics) for behavioral reflex to object movement; Magnetic Resonance Imaging (MRI) for in situ immune cell tracking and structural anatomy; histopathology for immune activation and neuronal cell degeneration, and immunoassay arrays for cytokine / chemokine levels. Overall, our objective is to provide extensive data that will identify translatable therapies targeting blast-induced vision impairments sustained by Warfighters. Thus, these studies may provide high impact advancements to the field of Military Medicine that can help lessen the burden experienced by thousands of severely visually impaired service members and their families, which can spill over into improving civilian medicine. Treatment and rehabilitation of visual system injuries in veterans has annually cost the US economy billions of dollars, and an enhanced understanding of etiology and effective treatment strategies could help with reducing a significant portion of this financial predicament. Thus, the proposed research has enormous benefit to supporting the mission and goals of the US Military.

- **KEYWORDS:** Rat, blast wave, neurotrauma, neurodegeneration, eye, retina, brain, inflammation, macrophage, microglia, astrocyte, neuron, omega-3 polyunsaturated fatty acid, electroretinography (ERG), visual acuity, optokinetics, magnetic resonance imaging (MRI), histopathology, immunoassay array, and cytokines.
- **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

- **What were the major goals of the project?**

The major goals for the project as detailed in the statement of work were as follows:

- 1) Obtain a WRAIR-IACUC and USAMRMC-ACURO approved animal use protocol for the study.
- 2) Place rats on an omega-3 fatty acid deficient diet for one month, as given alone or with fish oil supplementation administered by daily oral gavage.
- 3) Give the rats baseline visual acuity and ERG examinations and then expose at least half of them to double blast over pressure waves (1 min interval) in a compressed air driven shock tube. The remaining animals are utilized as shams (i.e., uninjured controls).

- 4) Repeat visual acuity and ERG exams on all rats at 3, 7, and 28 days post-exposure (ideally, n = 22). An additional time point of 14 days out was added to the study.
- 5) Subsets of rats are euthanized at 3, 7, and 28 days post-exposure (ideally, n = 18) and perfused with saline and then paraformaldehyde. Whole fixed carcasses (ideally, n = 8) are sent to our outside collaborators at the University of Pittsburgh's Animal Imaging center (Dr. T. Kevin Hitchens' lab) for MRI analysis (immune cell tracking and structural anatomy) of the eyes (retinas) and brains, which are eventually removed and returned to us. Alternatively, fixed eyes and brains are directly collected here from some perfused animals (ideally, n = 10).
- 6) Fixed eyes and brains (ideally, n = 18) are submitted to the local company FD Neurotechnologies for histopathology processing, i.e., H&E and silver stains, and CD68, GFAP, and Iba-1 immunohistochemistry. Returned slides are assessed under a microscope for immune cell activation and neuronal cell degeneration in the retina and brain visual centers.
- 7) Other subsets of rats are euthanized at 3, 7, and 28 days post-exposure (ideally, n = 4) and fresh plasma, retinas, and brains are collected. These tissues are then subjected to immunoassay arrays to determine the levels of various cytokines that are present.
- 8) Wrap up all sample and data processing and begin writing of publications for submission to US Army approved scientific journals. Submit quarterly and annual reports as required along the way of the study.

In summary, the four basic goals covered in the years 1 - 3 of the project were as follows:

Goal #1: Obtain an approved animal use protocol.

Goal #2: Report data for blast wave induced retina and brain inflammation at 3, 7, and 28 days post-exposure (i.e., ERG, visual acuity, MRI, histopathology, and cytokine assays).

Goal #3: Report data for shams, i.e., non-injured controls, at 3, 7, and 28 days post-exposure to mock blasting (i.e., ERG, visual acuity, MRI, histopathology, and cytokine assays).

Goal #4: Give conference presentations of the results and initiate their publication in appropriate peer reviewed scientific journals.

- **What was accomplished under these goals?**

Year #1: During the first year of the study, we had fallen 5 -6 months behind schedule due to problems obtaining the animal protocol and initiating the CRADA agreements to do the MRI analysis work on the animals with our collaborators, Dr. T. Kevin Hitchens' group at Carnegie Mellon University. He took a new position at the McGowan Institute's Animal Imaging Center at the University of Pittsburgh; and, thus, we had to resubmit the contract agreements and wait until he had moved into his new laboratory to send samples for analysis. This delay lead to a reset of the SOW schedule to the goals described under the first year, which were to accomplish gathering data primarily at 3 and 7 days post-blast (i.e., ERG, visual acuity, MRI, histopathology, and cytokine assays). Likewise, conference presentations were not possible during this time period, since no experimental data had been accumulated.

Year #2: In the second year of the study, we focused on moving the goals of obtaining data at 3 and 7 days post-blast forward to completion. Likewise, we pushed ahead our original plans of doing rats at a 28 day post-blast time point along with corresponding shams into a one year no cost extension period (i.e., 3rd year). In greater detail, during the second year, we brought in 8 experimental groups of animals (n = 8 - 18) for a grand total of 100 animals utilized. These animals were placed for at least one month on a continuous diet deficient or enriched in omega-3 polyunsaturated fatty acids. The division of animals within each group between the two diets was always an even 50/50 split. We provided placebo (soybean) and ocean fish oil

supplementation to the rats on the omega-3 deficient and enriched diets, respectively, as given once daily by oral gavage during at least 6 days of the week. Daily body weights were recorded for all of the animals. Blood glucose levels were also checked once per week for 20 animals out of each dietary treatment group. After one month of feeding the rats were exposed under anesthesia once to double blast over pressure waves (1 minute interval; 20 psi) using a compressed air driven shock tube, with some unexposed rats serving as shams. Also, following the exposures we recorded the rat's righting reflex, i.e., time to return to full consciousness from anesthesia, as an indicator of resulting brain injuries. Visual acuity exams (i.e., optokinetics) and electroretinography exams were carried out on the rats at least several days prior (baseline) to blast exposure and out to 3 and/or 7 days thereafter on 17 and 24 of the placebo and fish oil treated blast survivors or shams. All rats were euthanized at these respective two end points; and fresh tissues (retinas, brain, and liver) or paraformaldehyde perfused tissues (eyes and brains) and intact carcasses were collected. Terminal blood drawn from every rat was submitted for complete blood cell counts (CBC) and chemistry panel work up. We performed fatty acid composition analysis of liver samples collected from 5 placebo and 5 fish oil treated animals at 7 days post-blast, using lipid extraction and GC/MS methods. Cytokine concentration determinations, using multiplex immunoassay arrays, were done on collected fresh brains (mid-brain and cerebellum regions) and plasma for 24 placebo and 20 fish oil treated rats as shams or at 3 and 7 days post-blast. Histopathology (H&E and silver stains) and immunohistochemistry (GFAP, Iba-1, and CD68) assessments for activated immune cells and neuronal cell degeneration were performed on sections of fixed eyes (retina) and brains (optic tracts) taken from 5 & 5 placebo and 4 & 4 fish oil treated rats as shams or at 3 and 7 days post-blast. Magnetic resonance imaging (¹H- and ¹⁹F-MRI) was done on fixed whole carcasses to determine the structural damage and inflammation status of the eyes and brains for 8 placebo and 8 fish oil treated rats as shams or at 3 days post-blast. One amendment was done to the animal protocol to add a surgical technique required for doing advanced ERG recordings (visual evoked brain potentials), i.e., head screw implantations, and to add personnel (one main technician). Finally, we presented all of this work and the resulting preliminary data in poster format at three conferences held within the continental USA.

Year #3: In the third year of the study, which was an approved "no cost" extension period, we focused on moving ahead the goals of obtaining data at 7 and 28 days post-blast forward to completion. In greater detail, during the third year, we brought in another 8 experimental groups of animals (n = 10 - 30) for a grand total of 128 animals utilized. As previously described, these animals were split between being raised for one month on an omega-3 deficient or enriched diet; exposed to double blast over pressure waves or taken as shams; and out to 3, 7, and/or 28 days later subjected to outcome measures of body weight, righting reflex, visual acuity, ERG, blood work (CBC and chemistry panel), fatty acid analysis (liver and brain), histopathology & immunohistochemistry (retina and brain optic tracts), cytokine immunoassay arrays (plasma, brain, and retina), and MRI scanning (eye and brain). Body weights recordings were done on all of the animals prior to blast, i.e., 64 placebo and 64 fish oil treated. We also continued recording the body weights of the shams and blast survivors, especially those that went out to 28 days post-exposure. Righting reflex from isoflurane anesthesia was done on all of the shams and immediate blast survivors, i.e., 48 placebo and 52 fish oil treated animals. Visual acuity and electroretinography examines were done on 17 placebo and 15 fish oil treated blast survivors or shams at baseline and 3, 7, 14, and/or 28 days post-exposure. All of these rats were euthanized at end points of 3, 7, or 28 day post-exposure and eyes (retinas) and brains collected for subsequent neuropathological analysis. Terminal blood work was also done on all of these animals. Fatty acid composition analysis was done on the livers and brains from 29 placebo and 30 fish oil treated rats as shams or at 3, 7, or 28 days post-blast. Some of the livers had been already analyzed in year 2 of the study for these rats, i.e., 5 and 5 animals, respectively. Cytokine concentration determinations were done on retinas and brains from 15 placebo and 11 fish oil treated rats at 3, 7, and 28 days post-blast. Determinations for levels of cytokines of shams had been completed in year 2 of the study. Histopathology and immunohistochemistry were performed on eyes (retinas) and brains (optic tracks) of 21 & 10 placebo and 23 & 12 fish oil treated rats as shams or at 3 or 7 days post-blast. MRI scanning (structural anatomy and macrophage cell tracking) was done on 20 placebo and 21 fish oil treated rats as shams or at 3, 7, or 28 days post-blast. Two amendments were done to the animal protocol to add more rats and personnel (three back up technicians). Also, final tallies for all rats and outcomes done over the 3 years of the study are shown below in Table 1. Finally, we presented all of this work and the resulting preliminary data in poster format at three conferences held within the continental USA, as well as submitted an illustrated abstract to the USAMRMC for their FY17 Report to the Executive Agent (EA) on the Science and Technology Efforts and Programs Relating to the Prevention, Mitigation, and Treatment of Blast Injuries.

Table 1: Final tallies of animals fully reported for treatments and outcome measures.

Treatments	Visual acuity	ERG	Blood work	Retina / brain / plasma cytokines	Liver / brain fatty acids	Retina histo.	Brain histo.	MRI
Placebo; sham	6	6	15	3 / 9 / 9	5 / 5	9	5	8
Fish oil; sham	7	7	16	3 / 6 / 6	6 / 6	8	5	8
Placebo; 3 days post-blast	20	20	23	10 / 11 / 11	8 / 8	12	5	11
Fish oil; 3 days post-blast	18	18	25	10 / 10 / 10	8 / 8	12	4	12
Placebo; 7 days post-blast	17	17	22	8 / 9 / 0	8 / 8	5	5	8
Fish oil; 7 days post-blast	19	19	25	10 / 10 / 0	8 / 8	7	7	8
Placebo; 28 days post-blast	11	11	19	11 / 11 / 0	8 / 8	0	0	8
Fish oil; 28 days post-blast	10	10	18	10 / 10 / 0	8 / 8	0	0	8

Table 1. Numbers of animals are shown that were finally completed for each treatment and outcome measure, across the entire study. Total input of rats toward shams or double blast exposure for the placebo and fish oil groups was 100 subjects for each diet. This count encompasses all rats utilized in the study including those assigned to specific outcome measures, e.g. MRI. Prior to and/or during blasting, all animals were used for reporting extra outcomes of body weight gains, survival rates, righting reflex, and blood work. Plasma cytokines were only assessed at 3 days post-blast. Likewise, histopathology was not done on any retinas and brains at 28 days out. Collected samples, however, are available in storage for eventually carrying out both outcomes measures, if the data is required for future publications.

Detailed Experimental Methods, Results, and Conclusions:

I. Animals and Dietary Manipulations (Omega-3 Deficient and Enriched Diets):

Materials and Methods:

Adult male Sprague-Dawley rats are obtained from Charles River Labs (Portage, MI). This strain compared to others shows better long term recoveries from neuronal injuries. Initial body weights are ~ 100 g (30 days old) to help limit their final body sizes at our experimental end points. Rats are housed in our animal facility, under tightly regulated environmental conditions. Dietary requirements are provided by custom made chows as detailed below. One month prior to double blast wave injuries and continued thereafter until the experiment's end, rats are fed ad libitum a base diet that is deficient in long chain omega-3 polyunsaturated fatty acids. The diet is a commercial rodent chow that is essentially devoid of all long chain omega-3s including docosahexaenoic acid (DHA) (TestDiet®; "Typical American diet" - #: 5TLN; Purina Mills, LLC). The major goal of feeding the rats an omega-3 deficient diet is to induce a pro-inflammatory state, to aid in seeing changes in our outcome measures post-blast. In accordance with this, the "American" diet contains very high amounts of linoleic acid (LA; 8% of calories) an omega-6 fatty acid that is the precursor to inflammation stimulating arachidonic acid and its prostaglandin / leukotriene metabolites. We have shown previously and here (see below) that feeding this diet to the rats for one month depletes their liver stores of DHA by 65%; however, due to fatty acid conservation mechanisms, the brain DHA remains relatively unperturbed. During the feeding regime, the rats are scale weighed daily to monitor body weight gain. Also, their glucose levels are examined once per week (i.e., Fridays) by a handheld blood glucose monitor (Contour®, Bayer), using a small drop of blood that is drawn via needle prick of the tail.

Rats receive dietary omega-3 fatty acid supplementation using ultra-pure grade fish oil (ProOmega®; Nordic Naturals, Inc). The fish oil contains DHA at 23% by weight; and is given to the rats once daily by oral gavage at a DHA dose of 200 mg/kg/d. This amount of DHA is 4 times the USA-FDA recommendation for normal humans and 2 times that suggested by physicians during recovery from traumatic brain injuries. The fish oil also provides 273 mg/kg/d of eicosapentaenoic acid (EPA; an omega-3); which is converted to DHA and similar anti-inflammatory metabolites. Rats kept on the omega-3 deficient diet receive a placebo preparation (Nordic Naturals, Inc), consisting of soybean oil plus all additives found in the fish oil (i.e., flavors and preservatives). To prepare the fish oil for oral administration, an aliquot appropriate for DHA dosing the rat's body weight (~ 1 ml/kg) is combined with at least 0.5 ml of room temperature instant non-fat milk (10% wt/vol; Carnation - Nestlé). Placebo oil is given using identical volumes. The oil-milk mixture is rapidly agitated and then given to the rat by gavage directly into the stomach, using a 1 cc syringe fitted with a disposable plastic feeding tube (Harvard Apparatus). The procedure is done by finger collaring the rat, sliding the feeding tube down the throat / esophagus, and quickly depressing the syringe plunger. This method causes minimal discomfort and no harm to the animal.

Results and Conclusions:

Over the entire study, we fully raised a total of 200 rats for one month on an omega-3 fatty acid deficient or enriched diet (i.e., placebo and fish oil treated, respectively; 100 animals each). During the feeding regime we carefully monitored their body weights each day. Shown in Figure 1 is a bar graph of the starting and final body weights for the placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 100 and 100, respectively). We consistently found that there were absolutely no significant differences or trends detected between the two dietary groups in body weight gain over a one month feeding period, with an average mass increase of 9 g/d or 2%/d. Thus weight gain in rats is apparently a very analogous measure, with dietary omega-3 fatty acids playing no influence. This is an interesting finding, since omega-3 fatty acids are known to upregulate genes, via PPAR transcription factors, that are involved in the energy metabolism of fat stores. Our extremely large group sizes solidifies the validity of these contrary findings. Also, shown in Figure 2 is a bar graph of the weekly blood glucose levels checked for the placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 20 each). There were no significant differences in glucose levels detected between the two diets at any week during feeding over one month. Both dietary groups stayed well within the normal glucose range for rats, which we rigorously found from "house" chow fed rats to be 105 ± 13 mg/dL (dotted line; n = 14). These results satisfactorily prove that our base diet (high fat and carbohydrate with no omega-3s) does not generate insulin imbalance problems in the rats, i.e., pre-diabetic state, which might exacerbate neuronal injuries. Thus, we discontinued the weekly monitoring of the rat's blood glucose levels for the remainder of the study; which, in turn, spared the tail veins from damage that might impede the intravenous administration of contrast agent during the MRI assessments.

Figure 1: Body weight gains of rats over one month on dietary treatments.

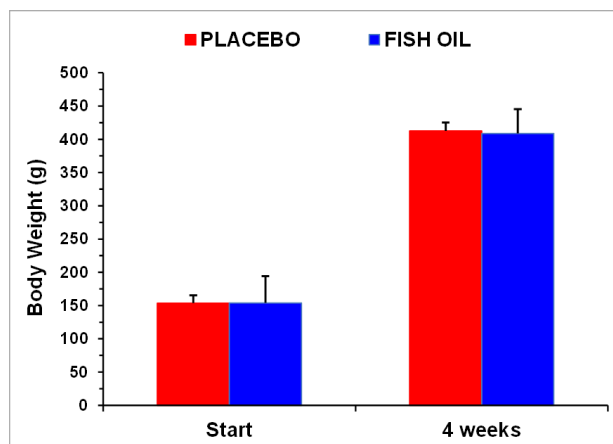


Figure 1. Bar graph for the starting and final body weights of the placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 100 and 100, respectively; mean $\pm$ SD). There were no significant differences

detected between the two dietary groups in body weight gain over a one month feeding period, with an average body mass increase of 9 g/d or a 2%/d.

Figure 2: Blood glucose levels of rats over one month on dietary treatments.

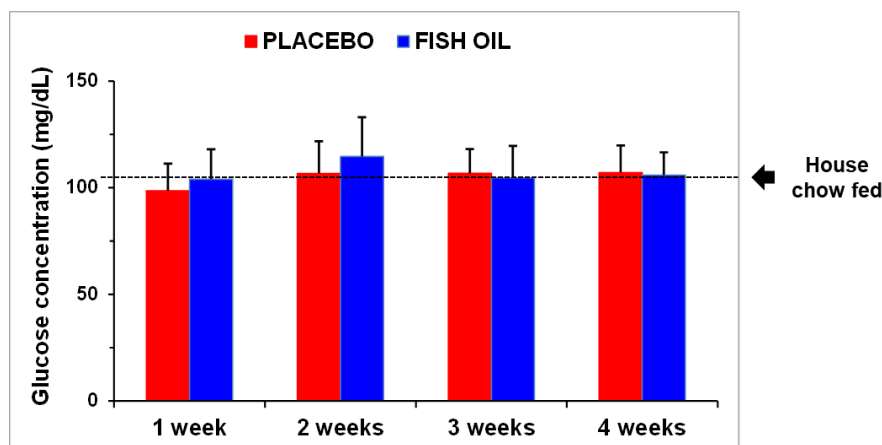


Figure 2. Bar graph for the weekly blood glucose levels of the placebo versus fish oil treated rats (placebo = red and fish oil = blue; $n = 20$ each; mean $\pm$ SD). Dotted line shows average glucose levels of normal “house” chow fed rats (105 ± 13 mg/dL; $n = 14$). There were no significant differences detected between any of these three dietary treatment groups.

II. Induction of Eye and Brain Injuries using Exposure to Double Blast Waves.

Materials and Methods:

After at least one month of dietary treatments (i.e., omega-3 deficient and enriched diets; placebo and fish oil, respectively), rats are placed under brief anesthesia using isoflurane gas. Anesthetized animals are placed in a prone transverse position inside a nylon mesh sling that is firmly secured to a metal frame sled. Rats are positioned with the left side of body align perpendicular to the inside of the sled, and hence right eye facing outward to the oncoming blast wave during exposure. In this manner, the left eye should serve as a control, expected to incur less severe injuries or none. The rat-loaded sled is inserted down the barrel of a compressed air driven shock tube to a preset position in its forward expansion chamber. The unawake animal is then exposed to two closely-coupled repeated blast over pressure waves (20 psi total pressure, 1 min interval), which has been previously shown by us to produce significant neuronal injuries to the retina and brain visual centers. The double blast waves are generated and propagated down the shock tube by a rapid-buildup compressed air rupturing of a Mylar membrane, of predetermined thickness to deliver 20 psi of air to the rat’s position, as clamped between the rear compression and forward expansion chambers. Blast waves travel by the rat with a Mach 1.34 shock front, 62 μ sec rise time, 8 msec duration, 126 m/s wind speed, and an acceleration g-force of > 1000 g. Double blasted rats are immediately removed from the shock tube and monitored during recovery. Animals exhibiting stable respiration are returned to their housing cages and laid on their sides. Time for the animal to regain an upright prone posture (i.e., righting reflex) is recorded, as an indication of its return to consciousness. Blasted rats are subjected at 3, 7, 14, and 28 days post-injury to blood work, visual acuity, ERG, fatty acid composition, and histopathology outcome measures, as to be described below.

Results and Conclusions:

Over the entire study, we exposed a total of 95 placebo and 96 fish oil treated rats to double blast over pressure waves. Shown in Figure 3 is a table of the long term survival numbers and a bar graph of the righting reflex (i.e., time to regain an upright posture) for the placebo versus fish oil treated rats, following blasting (placebo = red and fish oil = blue, $n = 95$ and 96 and 73 and 79 , respectively). Overall, we found the

incidence of blast related deaths (immediate and delayed combined) for the placebo versus fish oil treated rats was 29 and 27%, respectively; suggesting omega-3 fatty acid deficiency does not impact the animal's resiliency to blast. We did chi square analysis on the survival numbers and confirmed that there was no difference, despite the very large group sizes of nearly 100 rats per diet. Likewise, no significant differences were detected between the two dietary treatments for righting reflex post-insult (right graph); and there was only a slight trend (13%) for the fish oil treated rats to regain consciousness faster. The righting reflexes of both dietary treatment groups are consistent with rat having a concussive head injury induced disruption of brain activity. Indeed, for both groups, several animals that displayed a delayed death (> 3 hours) from blast-injury complications took a prolonged time to regain consciousness, i.e., > 20 minutes. We were also interested if the dietary treatments had an impact on the animal's general ability to physiologically recover over time following blast exposure. Shown in Figure 4 are line graphs of their body weight gains over 28 days following blast, as expressed as absolute weight (grams) and a percentage of baseline, for the placebo versus fish oil treated rats (n = 18 and 19, respectively). While there is not a significant difference in speed or amount of weight recovery across time between both diets, there is a continual trend for the fish oil group to attain a greater body size. Interestingly, area under the curve analysis showed the fish oil treated blasted animals to be significantly larger overall than their placebo counterparts. A side feature to come out of the weight gain curves is the presence in both diets of a substantial drop in body mass (~ 6%) out to 2 - 3 days post-blast, which thereafter showed an average linear rebound rate of 5.9 g/d ($r^2 = 0.994$) up to 28 days. The corresponding daily weight gain just prior to blast (i.e., over 4 days) as averaged for both diet groups was 8.5 g/d ($r^2 = 0.998$), which represents a 31% permanent loss in the animal's growth capacity. One potential cause for this deficit would be blast damage to the rat's digestive system (e.g. intestines) leading to malabsorption of nutrients and hence a reduced caloric intake. Alternatively, blast exposure in the rats could result in an imbalance of metabolic hormones effecting hunger and/or energy utilization (e.g. glucagon and insulin), by damage to the endocrine system (e.g. pancreas) or associated brain centers (e.g. hypothalamus).

Figure 3: Survival rates and Righting Reflex of rats following double blast exposure.

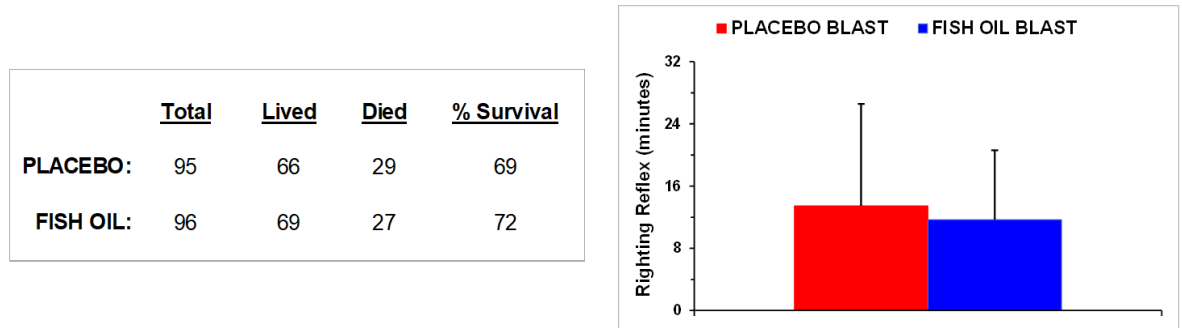


Figure 3. Bar graph and table for the survival rates and righting reflex of the placebo versus fish oil treated rats (placebo = red and fish oil = blue; n = 95 and 96 and 73 and 79, respectively; mean $\pm$ SD) shortly following double blast exposure. There were no significant differences between dietary treatment groups, as by chi square and t-test, respectively.

Figure 4: Body weight gains of rats following double blast exposure.

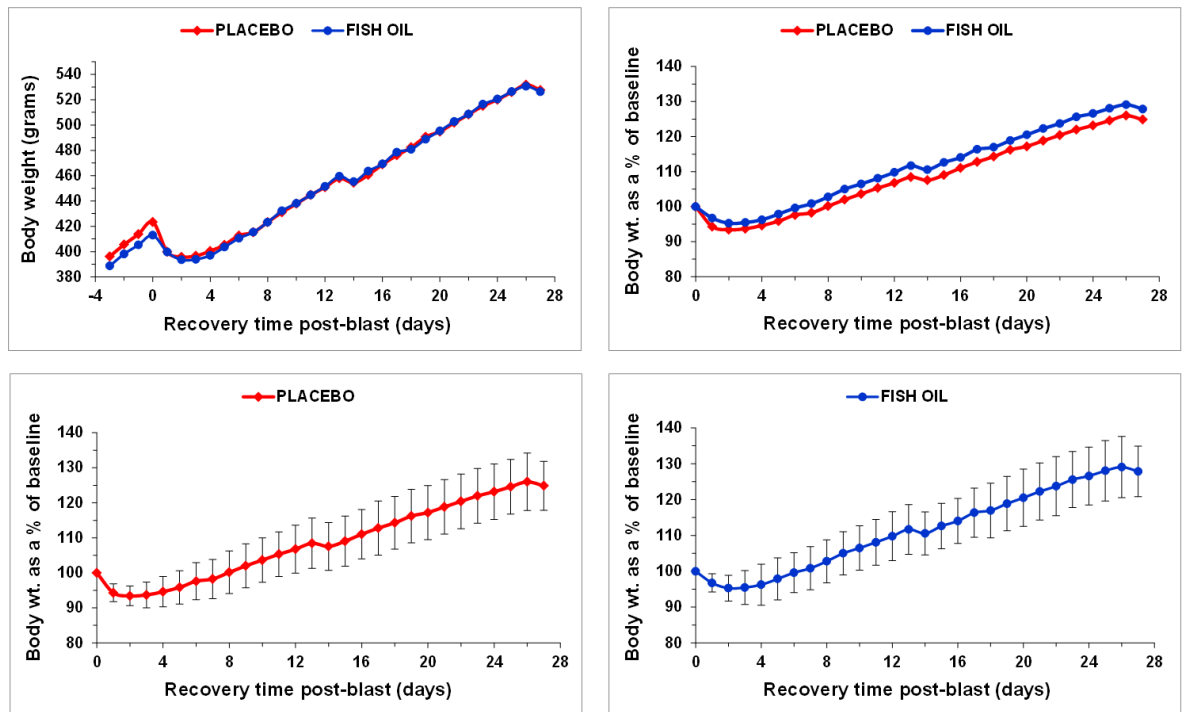


Figure 4. Line graphs for body weights of the placebo versus fish oil treated rats over 28 days following double blast wave exposure (placebo = red and fish oil = blue; $n = 19$ and 18 , respectively). Data is expressed as weight (grams) or a percentage of baseline (mean alone or mean $\pm$ SD). There was no significant difference detected between the two dietary groups in their body weight recoveries post-insult.

III. Visual Acuity (Optokinetics) Assessments of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

To judge their visual discrimination behavior capacities rats undergo visual acuity examinations using an optokinetics device (Optomotry unit; Cerebral Mechanics Inc). Measurements are taken at baseline at least two days prior to double exposure and at 3, 7, 14, and 28 days thereafter. To do the test the conscious rat is placed unrestrained on a 12 cm diameter pedestal raised 20 cm above the floor inside a 45 x 45 x 45 cm chamber, where it is surrounded on all four sides by LCD monitors that project a virtual rotating vertical black and white bar pattern having an adjustable spatial frequency (i.e., sine wave grating in cycles/degree). Flickering movement of the bar pattern immediately induces an eye tracking reflex (nystagmus) in the animals, which is viewed by an overhead mounted video tracking camera connected to an external computer with monitor. According to the manufacturer, a cursor is placed on the rat's forehead to keep the rotation of the cylinder centered according to the animal's perception, thereby "clamping" the effective spatial frequency of the grating. Visual acuities can be obtained in minutes in animals with no previous exposure to the task, and measurements can be repeated regularly even within the same day. System calibration, video capture, and an array of psychophysical testing methodologies (e.g., acuity and contrast thresholds) are managed by the instrument's controlling software.

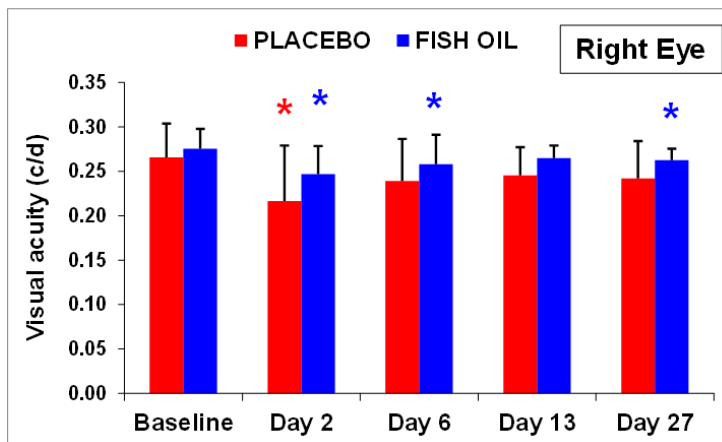
For assessment of visual acuities, rotation of the bar pattern is set to a fixed spatial frequency, at which normal rats can readily see (e.g., 0.04 cycles/degree at 100% contrast) and the rotation speed is increased stepwise to narrow the apparent width of the bars. Contribution of each eye is resolved by driving the pattern's rotation in opposite directions, i.e., clockwise and counter clockwise for left and right eyes, respectively. Eye pursuits are judged by watching for reflexive movements (side flicks) of the entire head, which are aligned with the direction of the stimulus rotation. Visual acuity thresholds are manually found by

steadily increasing over 15 min the spatial frequency of the bar pattern, until the animal no longer exhibits head tracking movements. The instrument helps the user reliably lock on the acuity thresholds by keeping the current spatial frequency hidden and using up and down speed reiterations in line with “no” and “yes” responses for head tracking movements via the computer’s key board. The test ends when the user inputs consistent responses of “no” for head tracking, and the acuity threshold is revealed. In this manner, the acuities are measured with an accuracy of 0.001 cycles/degree.

Results and Conclusions:

Over the entire study, we completed baseline and post-injury visual acuity assessments for a total of 37 placebo and 39 fish oil treated rats at 2 - 27 post-blast exposure. We also tested 6 placebo and 7 fish oil treated shams. We had to shift the original planned times back one day (e.g. 3 to 2 days), due to inability to do the visual acuity and ERG testing on the same day. Shown in Figure 5 are separate bar graphs for the right and left eye visual acuities (top and bottom panels) of placebo versus fish oil treated rats, at 2, 6, 13, and 27 days following double blast exposure (placebo = red and fish oil = blue, n = 37, 20, 17, 11, & 11 and 39, 18, 19, 10, & 10, respectively). Evaluation of separate shams found that there was no impact alone of the two diets on visual function; where we obtained combined eye acuities that were well within normal values reported for Sprague Dawley rats of 0.285 ± 0.021 and 0.286 ± 0.010 c/d (n = 6 and 7), respectively (graphs not shown). For both dietary treatment groups, however, there was a significant decline in their acuities for both the right and left eyes from baseline values (6 - 19% and 8 - 11%, respectively) at 2 - 6 days post-blast. The right eyes of the fish oil treated rats also showed significant acuity deficits (5%) that persisted at 27 days, but this impairment was present as a greater trend in the placebo group (9%). Interestingly, for the right eyes the pattern of recovery back towards baseline values was more pronounced in fish oil treated rats. Likewise, only for the placebo group was there was a trend for the acuity impairments over time to be greater (2 - 11%) for the right eye compared to left, which faced the blast. This suggests dietary omega-3 fatty acids may slightly help expedite the restoration of visual function following blast injury. Visual acuity response, however, is a complex mechanism involving many eye and brain structures working in concert; thus, these finding must be verified by ERG (electroretinography) examinations of retinal signaling output to see if they had a direct impact on alleviating neuronal damage in this tissue.

Figure 5: Visual acuity values of double blasted rats at 2 - 27 days post-exposure.



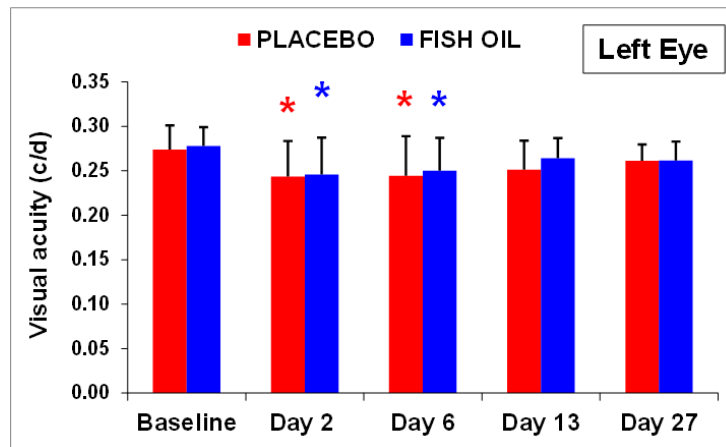


Figure 5. Bar graphs for the right and left eye visual acuities (top and bottom panels) of placebo versus fish oil treated rats at their respective baselines and from 2 - 27 days following double blast (placebo = red and fish oil = blue; $n = 37, 20, 17, 11, \& 11$ and $39, 18, 19, 10, \& 10$, respectively; mean $\pm$ SD). * $p < 0.05$, significant difference from baseline, as by t-test. There were no significant differences between the dietary treatment groups. Likewise, sham placebo and sham fish oil rats had combined-eye base acuities of 0.285 ± 0.021 and 0.286 ± 0.010 c/d ($n = 6$ and 7 ; mean $\pm$ SD), respectively.

IV. Electroretinography (ERG) Recordings of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

To determine the retina signaling response status of their eyes, at least 1 day prior to (baseline) and 3, 7, 14 and 28 days after double blasting, rats will undergo electroretinography (ERG) examinations, i.e., full field flash testing. This will be done using an ERG machine (Color Dome Full Field Ganzfeld unit; Diagnosys LLC). Full field flash ERG (standard ERG) detects the light stimulus evoked potentials arising from retinal photoreceptors and their synaptic joined bipolar and amacrine neurons. An ERG recording consists of two distinct waveform components, i.e., the initial a-wave (negative deflection) representing hyper-polarization of the photoreceptor cells, which results from a closure of their ion channels and turn off of neurotransmitter release, and a following b-wave (positive deflection and slow decay) arising from an opposite depolarization of the bipolar and amacrine neurons.

To prepare the rats for the ERG exams, they are first dark adapted overnight (16 hours) in a sealed darkroom to prime their retinal responses to light (scotopic ERG). We have found, however, that more robust and consistent responses are obtained, if possible, with an overnight adaptation (16 hours). All subsequent ERG procedures are carried out in the darkroom under red photography lights to which rat retinas don't respond; since they lack photoreceptors for red color vision. Each rat is placed under continuous isoflurane anesthesia, as delivered via a nose cone. The rat's pupils are then dilated using saline drops containing 0.5% tropicamide and 2.5% phenylephrine. As a corneal anesthetic, each eye receives 0.5% propracaine drops. The anesthetized animal is then laid prone on the exam table of the ERG machine. A ground electrode is fixed to the base of the tail, reference to each cheek, and recording to each eye's cornea. Our ERG machines use sub-dermally inserted pin electrodes (12 mm) for the ground and reference wires. The recording electrodes, which are fine gold-wire loops on movable arms, are situated directly onto the corneas using drops of 2% methylcellulose solution as a conductor and cushion. A light stimulus dome (LED flash lamp) is then lowered over the upper body of the animals. The rat is then presented with a series of white light flashes of increasing intensity at 0.1, 1, 3, and 10 cd.s/m² illuminations, 5 msec durations, and 30 - 60 sec intervals. The flashes are done in triplicate (2 sec intervals). These parameters are ideal for response detection of retinal photoreceptors involved in black and white vision. Results are reported as peak amplitudes of the a- and b-waves at the 10 cd.s/m² flash, which is a direct indication of retinal neuron health.

Results and Conclusions:

Over the entire study, we completed base line and post-injury ERG exams for a total of 35 placebo and 36 fish oil treated rats at 3 - 28 days post-blast exposure. Shown in Figure 6 are the bar graphs of the right and left eye a- and b-wave amplitudes (top & bottom and left & right panels, respectively), i.e., photoreceptor and bipolar/amacrine cell responses, for the placebo versus fish oil treated rats at 3, 7, 14, and 28 days following double blast exposure (placebo = red and fish oil = blue, $n = 35, 20, 17, 11, \& 11$ and $36, 18, 19, 10, \& 10$, respectively). The right and left eyes (retinas) of both dietary groups show significant decreases in a-wave amplitudes compared to their baseline values over 3 to 28 days out (i.e., 24 - 44%). Likewise, this pattern is highly reflected in the corresponding b-wave values (i.e., 17 - 40%), suggesting there was a loss of multiple retina neuronal cell types. While the losses of retinal signaling appear to be more pronounced in the fish oil treated group, their baseline starting values are significantly higher than that of the placebo animals (1.2-fold); which suggests omega-3 supplementation alone produced healthier retinas to start with. Evaluation of separate shams done for the fish oil and placebo groups agreed with these findings as well as produced similar ERG recordings ($n = 6$ and 7 ; graphs not shown). Also, by 28 days post-blast, the fish oil treated rats have undergone a greater rebound back towards baseline, with the right eye being significantly higher once again compared to the placebo group (1.3-fold). Shown in Figure 7, are bar graphs of the B-wave to A-wave ratios calculated from this same data; which is representative of the ability of the intact retina photoreceptors to transmit synaptic information onto the synaptic - interconnected bipolar / amacrine cells (inner nuclear layer). In this case, only the fish oil treated group has values that were significantly higher than baseline at almost every day examined post-blast (1.1 - 1.2-fold); whereas, the placebo group was trending to be lower over 3 and 7 days out. This suggests that the neuronal signaling is actually enhanced in the non-damaged retinal regions of the fish oil treated rats. Overall, our results demonstrate that blast exposure is markedly damaging the retinal neurons in both eyes; and omega-3 fatty acids are effective at alleviating this, especially at the chronic phase of injury. These findings are also consistent with the therapeutic benefits of fish oil that we previously noted for shielding visual acuity against losses from the blast exposure.

Figure 6: ERG amplitudes (a- and b-wave) of double blasted rats at 3 - 28 days post-exposure.

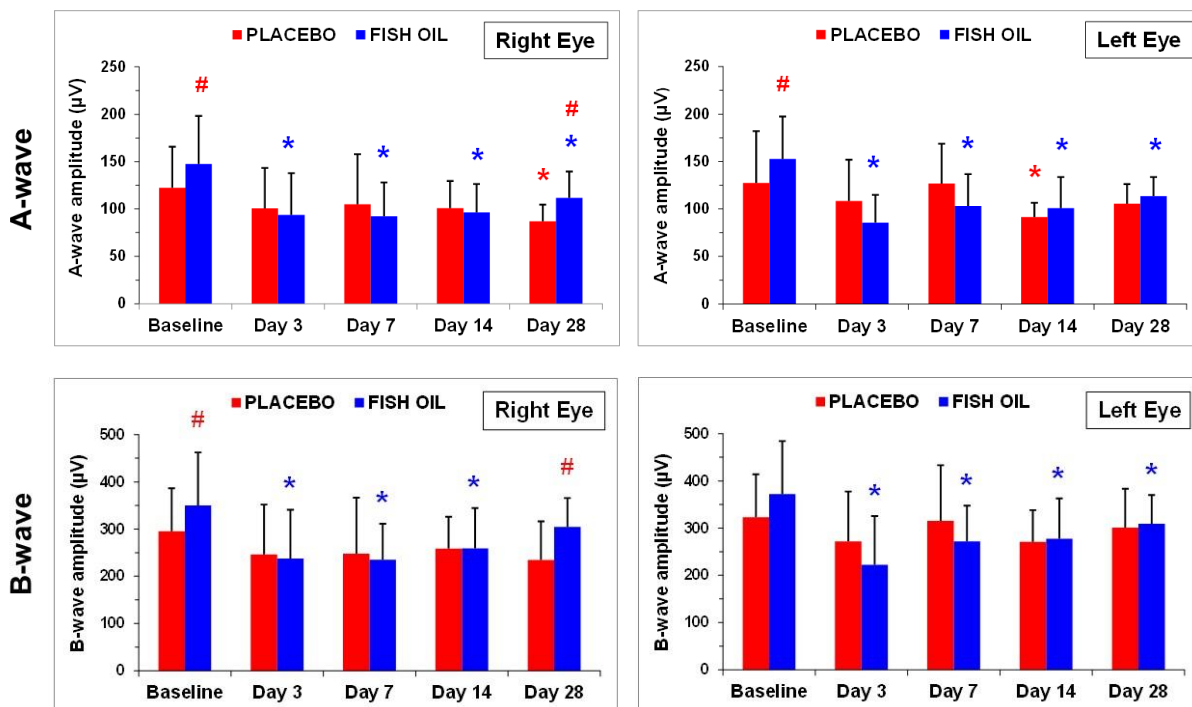


Figure 6. Bar graph for the right and left eye, a- and b-wave amplitudes (top & bottom and left & right panels, respectively) of placebo versus fish oil treated rats (placebo = red and fish oil = blue; $n = 35, 20, 17, 11 \& 11$ and $36, 18, 19, 10, \& 10$, respectively; mean $\pm$ SD) at baseline and 3, 7, 14, and 28 days following double

blast. ERGs were done using a 10 cd.s/m² flash stimulus. * $p < 0.05$, significant difference from baseline, as by t-test. # $p < 0.05$, significant difference between dietary treatment groups, as by t-test.

Figure 7: ERG B-wave to A-wave ratios of double blasted rats at 3 - 28 days post-exposure.

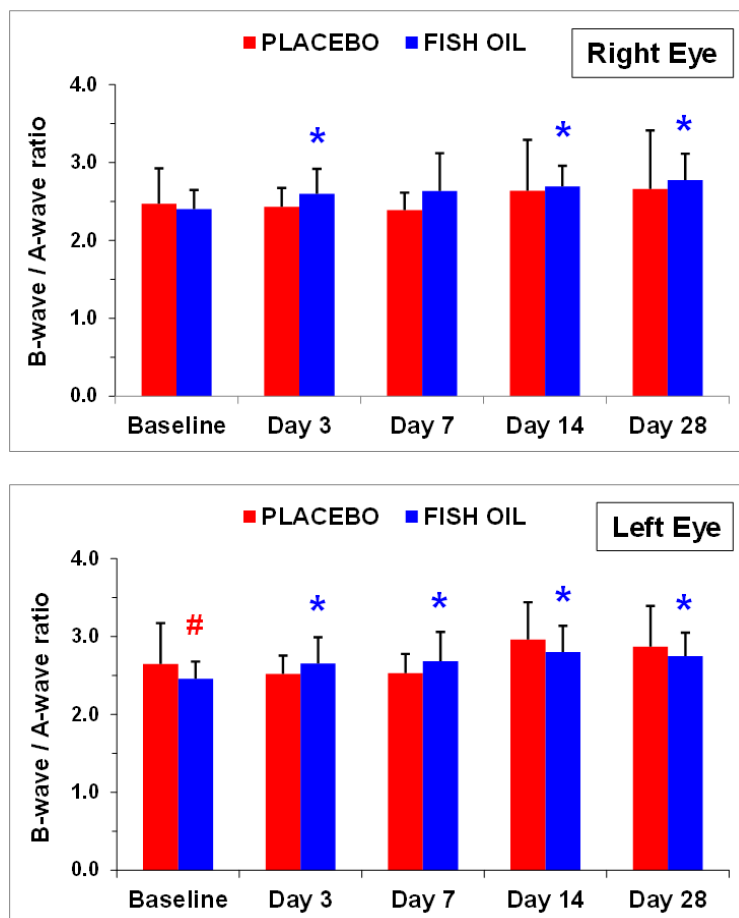


Figure 7. Bar graph for the right and left eye, b- to a-wave ratios (top and bottom panels) of placebo versus fish oil treated rats (placebo = red and fish oil = blue; $n = 35, 20, 17, 11$ & 11 and $36, 18, 19, 10$, & 10 , respectively; mean $\pm$ SD) at baseline and 3, 7, 14, and 28 days following double blast. * $p < 0.05$, significant difference from baseline, as by t-test. # $p < 0.05$, significant difference between dietary treatment groups, as by t-test.

V. Blood Work and Fatty Acid Assessments of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

Rats from the placebo and fish oil dietary treatment groups are euthanized at 3, 7, 14, and 28 days post-blast exposure for collection of fresh tissues, i.e., plasma, brain, and retinas. Animals will be deeply anesthetized by isoflurane inhalation and then subjected to terminal blood exsanguination, using cardiac puncture as done with a syringe through the chest. Excess blood is collected in EDTA and heparinized vacuum tubes kept on ice. Some whole blood is submitted to the WRAIR Department of Clinical Pathology for complete blood-cell count (CBC; hemocrit, platelets, and white blood cell types) and chemistry panel (cholesterol, triglycerides, alanine aminotransferase, and aspartate aminotransferase). The biomarkers selected for the chemistry panel are mainly a measure of liver function, in light of the high fat / low omega-3 base diet we are giving the rats. Remaining blood is centrifuged to obtain plasma. Once respiration and heartbeat have ceased, the rats are subjected to guillotine decapitation. Whole brains are removed from the heads, washed with saline, dissected

into cerebellum, cortex, and midbrain regions, and then quick frozen on dry ice. Eyes are enucleated and dissected to obtain the retinas. An eye cup is formed by cutting off the eye's anterior segment (i.e., cornea, iris, and lens) and the retina is isolated using jeweler's forceps and washed with saline. Retinas from both eyes are combined in one tube, and quick frozen on dry ice. A section of liver lobe, for fatty acid profile purposes, is also removed and quick frozen. All tissue samples are stored frozen at -80°C for future analysis.

For fatty acid analysis, liver samples are homogenized, by probe sonicator, into methanol containing butylated hydroxytoluene (BHT) as an antioxidant. Prior to homogenization, heptadecanoic acid (17:0) is added as internal standard for later quantification purposes. Total lipids are extracted from the tissue homogenates using chloroform, as partitioned out by addition of KCl solution. Chloroform phases containing crude total lipids are converted to fatty acid methyl esters (FAMES) by reaction with BF_3 in methanol. FAMES are extracted into hexane and then analyzed on an Agilent Technologies 5975C / 7890A gas chromatograph - mass spectrometer (GC/MS) system. Mass detector derived peaks (selective range, total ion count) are identified by retention time comparison with a standard mixture of FAMES, which includes all omega-3 and omega-6 polyunsaturated fatty acids of interest. These are also used to derive ionization efficiency response factors for each fatty acid. Concentrations of fatty acids in the original tissue sample (g per wet wt.) are determined by direct proportional comparison of their peak areas to that of the added 17:0 internal standard.

Results and Conclusions:

Over the course of the entire study, we finished the blood work, i.e., complete blood count (CBC) and chemistry panel, for a total of 79 placebo and 84 fish oil treated rats. Shown in Figure 8 is a table for the CBC and chemistry values of terminal blood for the placebo and fish oil treated rats as shams and at 3, 7, and 28 days following double blast (top and bottom panels, $n=15$ & 16, 23, 22, & 19, and 25, 25, & 18, respectively). Listed at the right side of the sham are the various hematological factors, along with their accepted ranges for normal rats, which were assessed, e.g., white blood cells (WBC), hemocrit (HCT), and triglycerides (TG). Those factors whose average is substantially above normal are highlighted light blue; and corresponding amount of abnormal animals are shown inside red brackets. Functionally, most crucial to our study are monocytes (MONO), which can transform into injured tissue destroying macrophages. Monocyte levels in both dietary treatments were elevated well beyond normal range at 3 - 28 days post-blast, but this phenomenon was also noted in the shams. There was, however, an apparent trend for the blasted fish oil groups to have slightly lower monocyte values across all time points. Eosinophils, however, were abnormally increased in both blasted dietary treatment groups at 7 days out and the blasted placebo animals at 28 days out, which are involved in heightened allergy responses, i.e., histamine release. Interestingly, across time, the total white blood cell and lymphocyte (infection response) counts have a trend to be lower in the blasted fish oil treated rats. Consistent with an injury response, however, there is a trend for both blasted dietary groups at all times to have increased in these immune cell counts compared to the sham values. Overall, these findings indicate that dietary omega-3 fatty acids have some therapeutic benefit against blast injury by impeding a systemic inflammatory response.

As also shown in the Figure 9 table below, triglyceride levels are elevated at in the shams and at 3 - 28 days post-blast for both dietary treatments. Likewise, cholesterol is elevated in the placebo groups, but only at 3 days post-blast. Surprisingly, in the shams and at 3 days post-blast the cholesterol and triglyceride levels were significantly lowered in the fish oil treated animals (23, 31, 20, and 25%, respectively), but this was evidently not impacted by the blast exposure. There are strong indications of liver problems in the animals, since the AST and/or AST / ALT ratio values were elevated for both dietary treatments, including shams, and at all end points, especially 28 days post-blast. This suggests the diets alone are causing liver problems, e.g. fatty liver from the base "Typical American Diet" chow, which is high in total fat content. Liver inflammation could also explain why blood lymphocytes are so greatly elevated at 28 days post-blast in the placebo treated group, since this organ is known to activate these immune cells. Fish oil treatment, however, significantly reduced the AST and AST / ALT ratio values to normal levels at 7 and 28 days post-blast (15, 19, 13 and 16%, respectively). Overall, these findings imply dietary omega-3 fatty acids have a positive benefit towards maintaining liver function and modulating immune cell responses. Overall, this may also underlie the ability of omega-3s to prevent an excessive accumulation of cholesterol and triglycerides in the blood (i.e. at 3 days out), which is a hallmark of their numerous reported cardiovascular benefits that could also assist the brain and retina in healing normal injuries due to improved blood / oxygen supply to these tissues.

We also determined the liver and corresponding brain fatty acid compositions from 29 placebo and 30 fish oil treated rats. Shown in Figure 9 are tables for the liver and brain fatty acid composition values of placebo versus fish oil treated rats, as from shams and at 3, 7, and 28 days following double blast exposure (n = 5, 8, 8, & 8 and 6, 8, 8, & 8, respectively). Content of each fatty acid is expressed as a percentage of the total. The most crucial of these to our study are the major biological omega-6 and omega-3 fatty acids: arachidonic acid (ARA; 20:4 ω -6), eicosapentaenoic acid (EPA; 20:5 ω -3), and docosahexaenoic acid (DHA; 22:6 ω -3). We found that in the livers of the fish oil versus placebo treated rats the levels of DHA and EPA were significantly increased across all time points measured (3 - 5 and 12 - 15-fold, respectively). This was reflected in a significant and highly consistent decrease of the liver's omega-6 to omega-3 and ARA to DHA ratios (4 flat and 4 - 6-fold, respectively), which are taken as strong indicators of inflammation potential status. While the percentage of liver DHA remained relatively constant in the placebo groups, they appeared to have small declines over time post-blast in the fish oil treated animals, suggestive of an injury catalyzed mobilization of liver DHA out into the blood circulation. Overall, these findings demonstrate that providing fish oil to the rats for one month dramatically boosted the omega-3 fatty acid index of their tissues for suppression of neuroinflammation processes. The abundant DHA in the fish oil treated animal livers would also be available for systemic release to the brain to help build new neuronal membranes. Thus, our fish oil treated rats should be more resilient to blast induced neuronal injuries. Interestingly, the levels of ARA in the liver were nearly identical between the two dietary treatment groups across all times monitored post-blast. Production of this omega-6 fatty acid should have been greatly diminished by the high omega-3s levels. ARA is also the direct precursor for generation of inflammation stimulating prostaglandins and leukotrienes. Thus, based on the liver data, it appears that the one month feeding period of fish oil that we used might not have been enough to reap the full anti-inflammatory and neuronal membrane building benefits of the increased tissue omega-3 fatty acid levels. In light of this, we also looked at the fatty acid compositions of the corresponding brains to see if the DHA levels had likewise increased in that compartment. As shown in Figure 9 (second panel), we found that there was a small but significant increase in the percentage of brain DHA for the fish oil versus placebo treated shams and those at 28 days post-blast (1.1 - 1.2-fold). Again, this was supported by nearly equivalent decreases in the associated ratios of omega-3 to omega-6 fatty acids across all fish oil groups over time. In this case, however, brain ARA significantly decreased in the fish oil rats at all time points post-blast (4 - 9%), which is indicative of DHA's suppressive action against inflammation mechanisms (e.g. prostaglandin production). The fact that we were able to attain significant changes in the brain fatty acids through dietary manipulations is highly impressive accomplishment in itself, since the brain is well known to have conservation mechanisms in place that prevent such alternations from readily taking place over a relatively short time frame (i.e., months versus years). We did not, however, get a chance due to low availability of collected tissue to look at the retina levels of ARA, EPA, and DHA to see if they are also shifting after fish oil supplementation in a favorable direction towards halting neuroinflammation.

Figure 8: Blood work of shams and double blasted rats at 3 - 28 days post-exposure.

	SHAM		SHAM		Normal Ranges:	
	PLACEBO (n = 15)		FISH OIL (n = 16)			
Blood Test	Ave	SD	Ave	SD		
WBC (per μ l):	7529	$\pm$ 2412	6577	$\pm$ 1748	WBC	3300 - 8700
NEUT (per μ l):	805	$\pm$ 315	773	$\pm$ 317	NEUT	300 - 1700
LYMPH (per μ l):	6196	$\pm$ 2273	5346	$\pm$ 1543	LYMPH	2600 - 7100
MONO (per μ l):	437 $\pm$ 111 (15)		375 $\pm$ 170 (14)		MONO	0 - 200
EO (per μ l):	90 $\pm$ 30		82 $\pm$ 39		EO	0 - 100
BASO (per μ l):	1 $\pm$ 4		1 $\pm$ 3		BASO	0 - 20
HCT (% vol):	41 $\pm$ 2		42 $\pm$ 2		HCT	33 - 45
PLT (per nl):	963 $\pm$ 142		896 $\pm$ 160		PLT	493 - 1124
CHOL (mg/dL):	93 $\pm$ 17 (6)		81 $\pm$ 18		CHOL	20 - 92
TG (mg/dL):	297 $\pm$ 105 (15)		206 $\pm$ 111 (15)		TG	27 - 108
AST (U/L):	87 $\pm$ 25		83 $\pm$ 17		AST	39 - 110
ALT (U/L):	43 $\pm$ 21		41 $\pm$ 14		ALT	20 - 61
AST / ALT:	2.2 $\pm$ 0.5 (9)		2.2 $\pm$ 0.7 (10)		AST / ALT	1.0 - 2.0

= above normal range
Red brackets are the amount of rats above normal
p < 0.05, placebo vs. fish oil, as by t-test

	BLASTED 3 DAYS POST		BLASTED 3 DAYS POST		BLASTED 7 DAYS POST		BLASTED 7 DAYS POST		BLASTED 28 DAYS POST		BLASTED 28 DAYS POST	
	PLACEBO (n = 23)		FISH OIL (n = 25)		PLACEBO (n = 22)		FISH OIL (n = 25)		PLACEBO (n = 19)		FISH OIL (n = 18)	
Blood Test	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
WBC (per µl):	7606	± 2178	6612	± 2512	7455	± 1735	7114	± 3468	8146	± 2587	7303	± 2835
NEUT (per µl):	970	± 775	876	± 664	946	± 357	1302	± 1502	933	± 480	917	± 413
LYMPH (per µl):	6096	± 1936	5246	± 1979	5875	± 1551	5211	± 2044	6712	± 2074	5928	± 2465
MONO (per µl):	463 ± 217 (20)		406 ± 197 (19)		494 ± 177 (21)		479 ± 342 (22)		381 ± 150 (18)		366 ± 124 (15)	
EO (per µl):	73	± 29	86	± 43	135 ± 174 (8)		116 ± 77 (13)		116 ± 50 (11)		89	± 51
BASO (per µl):	4	± 5	3	± 5	5	± 6	5	± 6	4	± 5	3	± 5
HCT (% vol):	40	± 5	41	± 2	40	± 2	40	± 2	40	± 2	# 39	± 2
PLT (per nl):	902	± 192	986	± 135	1049	± 164	1045	± 186	840	± 153	929	± 161
CHOL (mg/dL):	99 ± 25 (11)		# 80 ± 20		75 ± 11		70 ± 15		74 ± 18		72	± 15
TG (mg/dL):	157 ± 68 (16)		# 118 ± 53 (14)		201 ± 77 (19)		170 ± 63 (22)		254 ± 118 (16)		222 ± 92 (16)	
AST (U/L):	98	± 34	92	± 31	99	± 24	# 85 ± 21		118 ± 36 (9)		# 96 ± 18	
ALT (U/L):	46	± 12	52	± 17	45	± 9	44	± 13	48	± 13	47	± 7
AST / ALT:	2.1 ± 0.6 (11)		1.9 ± 0.5		2.3 ± 0.5 (15)		# 2.0 ± 0.3		2.5 ± 0.8 (15)		# 2.1 ± 0.4 (8)	

Figure 8. Tables for CBC and chemistry of blood from placebo versus fish oil treated rats for shams and at 3, 7, and 28 days following double blast (top and bottom panels; n =15 & 16, 23, 22, & 19, and 25, 25, & 18, respectively; mean ± SD). Listed at the far right side (top panel) are the hematological factors examined, along with their reported normal ranges, i.e., white blood cells (WBC), hemocrit (HCT), platelets (PLT), cholesterol (CHOL), triglycerides (TG), aspartate amino transferase (AST), alanine aminotransferase (ALT), and ratio of AST to ALT (AST / ALT). White blood cells are broken down into sub-types of neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), and basophils (BASO). Those factors whose average is substantially above normal range are highlighted in light blue; and amount of abnormal rats are shown inside red brackets. # p > 0.05 (highlighted yellow); significant difference between dietary treatment groups, as by t-test.

Figure 9: Liver and brain fatty acids of shams and double blasted rats at 3 - 28 days post-exposure.

LIVER													
		SHAM		3 DAYS POST		7 DAYS POST		28 DAYS POST					
		PLACEBO (n = 5)		FISH OIL (n = 6)		PLACEBO (n = 8)		FISH OIL (n = 8)		PLACEBO (n = 8)		FISH OIL (n = 8)	
Fatty Acids		Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
C14:0		1.6 ± 0.4		1.1 ± 0.2		1.2 ± 0.3		0.8 ± 0.3 #		1.2 ± 0.3		1.2 ± 0.2	
C16:0	Palmitic	19.6 ± 0.7		19.5 ± 1.2		18.2 ± 0.5		17.7 ± 1.0		18.8 ± 0.5		19.4 ± 1.2	
C16:1n-7		3.4 ± 0.9		2.5 ± 0.3		2.5 ± 1.2		1.6 ± 0.7		2.5 ± 0.7		2.7 ± 0.8	
C18:0	Stearic	6.8 ± 1.1		7.9 ± 1.8		8.0 ± 1.3		9.9 ± 2.3		8.5 ± 1.8		8.4 ± 2.0	
C18:1n-7	Oleic	26.7 ± 1.3		22.5 ± 1.3 #		25.2 ± 1.5		20.3 ± 2.4 #		24.9 ± 2.1		22.1 ± 2.4 #	
C18:2n-7		4.4 ± 0.4		3.5 ± 0.5 #		3.8 ± 0.7		2.7 ± 0.7 #		3.9 ± 0.5		3.4 ± 0.5	
C18:3n-6	LA	23.5 ± 1.4		21.5 ± 0.9 #		25.1 ± 3.5		22.2 ± 1.0 #		24.3 ± 1.7		21.5 ± 1.8 #	
C18:3n-3	GLA	0.3 ± 0.03		0.16 ± 0.03 #		0.3 ± 0.04		0.2 ± 0.04 #		0.3 ± 0.05		0.2 ± 0.03 #	
C18:3n-3	ALA	1.5 ± 0.31		1.5 ± 0.3		1.6 ± 0.4		1.3 ± 0.3		1.4 ± 0.31		1.4 ± 0.4	
C20:0		0.02 ± 0.002		0.02 ± 0.005		0.03 ± 0.01		0.02 ± 0.01		0.03 ± 0.01		0.03 ± 0.003	
C20:1n-9		0.6 ± 0.1		0.5 ± 0.2		0.5 ± 0.1		0.3 ± 0.2 #		0.5 ± 0.1		0.5 ± 0.1	
C20:3n-6	DHGLA	0.6 ± 0.3		0.7 ± 0.2		0.8 ± 0.1		0.7 ± 0.2		0.9 ± 0.1		0.9 ± 0.1	
C20:4n-6	ARA	8.6 ± 1.7		7.0 ± 1.6		9.4 ± 1.8		8.1 ± 1.9		10.0 ± 1.9		7.3 ± 1.9 #	
C20:5n-3	EPA	0.18 ± 0.07		2.2 ± 0.8 #		0.2 ± 0.07		3.0 ± 0.6 #		0.2 ± 0.03		2.4 ± 0.6 #	
C22:0		0.02 ± 0.005		0.02 ± 0.01		0.02 ± 0.01		0.03 ± 0.01		0.03 ± 0.01		0.03 ± 0.01	
C22:4n-6	DTA _{n-6}	0.32 ± 0.12		0.16 ± 0.05 #		0.5 ± 0.3		0.2 ± 0.05 #		0.4 ± 0.2		0.14 ± 0.05 #	
C22:5n-6	DPA _{n-6}	0.06 ± 0.02		0.04 ± 0.01		0.1 ± 0.05		0.05 ± 0.01 #		0.1 ± 0.04		0.04 ± 0.01 #	
C22:5n-3	DPA	0.41 ± 0.11		2.9 ± 0.9 #		0.5 ± 0.3		3.2 ± 0.9 #		0.4 ± 0.2		2.7 ± 1.0 #	
C24:0		0.04 ± 0.01		0.06 ± 0.01 #		0.03 ± 0.01		0.05 ± 0.01		0.05 ± 0.01		0.07 ± 0.04	
C22:6n-3	DHA	1.3 ± 0.3		6.2 ± 0.8 #		1.9 ± 0.3		7.7 ± 1.0 #		1.7 ± 0.3		5.5 ± 0.8 #	
C24:1n-9		0.03 ± 0.005		0.1 ± 0.02 #		0.04 ± 0.01		0.1 ± 0.03 #		0.04 ± 0.01		0.1 ± 0.03 #	
% DHA of total FA:		1.3 ± 0.3		6.2 ± 0.8 #		1.9 ± 0.3		7.7 ± 1.0 #		1.7 ± 0.3		5.5 ± 0.8 #	
DHA conc; mg/g:		1.0 ± 0.2		4.4 ± 1.2 #		1.4 ± 0.3		4.8 ± 1.3 #		1.0 ± 0.3		3.4 ± 1.3 #	
ω-6 / ω-3 ratio:		9.9 ± 1.0		2.4 ± 0.5 #		8.7 ± 1.2		2.1 ± 0.3 #		9.9 ± 1.7		2.6 ± 0.6 #	
ARA / DHA ratio:		6.5 ± 0.7		1.1 ± 0.3 #		5.0 ± 0.9		1.1 ± 0.2 #		6.2 ± 1.7		1.4 ± 0.5 #	

** DHA concentrations are expressed as mg per g wet wt. of tissue.
p ≤ 0.05, for placebo vs. fish oil group, as by t-test.

 = Omega-6 polyunsaturated fatty acid
 = Omega-3 polyunsaturated fatty acid

BRAIN	SHAM		3 DAYS POST				7 DAYS POST				28 DAYS POST			
	PLACEBO (n = 5)		FISH OIL (n = 6)		PLACEBO (n = 8)		FISH OIL (n = 8)		PLACEBO (n = 8)		FISH OIL (n = 8)		PLACEBO (n = 8)	
	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
Fatty Acids														
C14:0	0.2 ± 0.0		0.2 ± 0.0		0.2 ± 0.02		0.2 ± 0.02		0.2 ± 0.01		0.2 ± 0.01 #		0.2 ± 0.01	0.2 ± 0.01
C16:0	24.7 ± 1.2		24.5 ± 2.1		24.1 ± 0.4		23.4 ± 1.2		23.4 ± 0.8		23.2 ± 0.5		23.9 ± 1.0	24.3 ± 1.0
C16:1 ω -9	0.48 ± 0.02		0.51 ± 0.02 #	↑	0.5 ± 0.03		0.5 ± 0.03		0.5 ± 0.02		0.4 ± 0.02		0.5 ± 0.03	0.5 ± 0.04
C18:0	24.4 ± 0.4		23.9 ± 0.5		23.7 ± 0.3		24.3 ± 0.3 #	↑	24.2 ± 0.8		24.9 ± 0.4 #	↑	24.2 ± 0.5	23.8 ± 0.4
C18:1 ω -9	18.2 ± 1.3		17.5 ± 1.0		16.6 ± 0.4		16.9 ± 0.4		17.8 ± 1.1		18.4 ± 0.4		17.0 ± 0.5	17.4 ± 0.4
C18:2 ω -7	4.2 ± 0.2		4.0 ± 0.3		4.0 ± 0.1		3.9 ± 0.1		4.1 ± 0.2		4.0 ± 0.2		4.0 ± 0.2	4.0 ± 0.1
C18:2 ω -6	0.8 ± 0.1		0.9 ± 0.1		0.9 ± 0.1		1.0 ± 0.1		0.9 ± 0.1		0.9 ± 0.04		0.9 ± 0.1	1.0 ± 0.1 #
C18:3 ω -6	0.03 ± 0.01		0.02 ± 0.004		0.02 ± 0.004		0.02 ± 0.004		0.03 ± 0.003		0.03 ± 0.004 #	↑	0.02 ± 0.004	0.02 ± 0.003
C18:3 ω -3	ND		ND		ND		ND		ND		ND		ND	ND
C20:0	0.2 ± 0.03		0.2 ± 0.05		0.1 ± 0.03		0.2 ± 0.05 #	↑	0.17 ± 0.04		0.25 ± 0.04 #	↑	0.21 ± 0.04	0.15 ± 0.03 #
C20:1 ω -9	0.8 ± 0.2		0.7 ± 0.2		0.7 ± 0.1		1.0 ± 0.2 #	↑	0.8 ± 0.1		1.1 ± 0.1 #	↑	0.93 ± 0.19	0.75 ± 0.12 #
C20:3 ω -6	0.2 ± 0.03		0.2 ± 0.02		0.17 ± 0.03		0.22 ± 0.03 #	↑	0.17 ± 0.01		0.20 ± 0.02 #	↑	0.16 ± 0.02	0.20 ± 0.03 #
C20:4 ω -6	11.4 ± 0.3		11.0 ± 0.6		12.0 ± 0.3		11.2 ± 0.3 #	↓	11.6 ± 0.6		10.6 ± 0.4 #	↓	11.8 ± 0.3	11.3 ± 0.3 #
C20:5 ω -3	ND		ND		ND		ND		ND		ND		ND	ND
C22:0	0.07 ± 0.02		0.08 ± 0.03		0.07 ± 0.02		0.1 ± 0.04		0.1 ± 0.04		0.1 ± 0.02		0.09 ± 0.02	0.06 ± 0.02 #
C22:4 ω -6	2.0 ± 0.2		1.8 ± 0.3		2.4 ± 0.2		2.0 ± 0.2 #	↓	2.1 ± 0.4		1.7 ± 0.3 #	↓	2.52 ± 0.24	1.93 ± 0.27 #
C22:5 ω -6	0.4 ± 0.1		0.2 ± 0.05		0.5 ± 0.1		0.2 ± 0.1 #	↓	0.4 ± 0.1		0.1 ± 0.04 #	↓	0.52 ± 0.10	0.16 ± 0.05 #
C22:5 ω -3	0.05 ± 0.01		0.1 ± 0.02 #	↑	0.05 ± 0.01		0.1 ± 0.01 #	↑	0.05 ± 0.01		0.08 ± 0.01 #	↑	0.05 ± 0.01	0.08 ± 0.01 #
C24:0	0.1 ± 0.03		0.1 ± 0.04		0.1 ± 0.03		0.2 ± 0.07 #	↑	0.1 ± 0.05		0.2 ± 0.04 #	↑	0.13 ± 0.03	0.10 ± 0.02 #
C22:6 ω -3	11.4 ± 0.7		13.6 ± 0.3 #	↑	13.4 ± 0.5		13.7 ± 0.5		12.8 ± 1.1		12.6 ± 0.8		12.2 ± 0.8	13.6 ± 1.3 #
C24:1 ω -9	0.4 ± 0.08		0.5 ± 0.1		0.4 ± 0.09		0.7 ± 0.2 #	↑	0.5 ± 0.2		0.8 ± 0.1 #	↑	0.6 ± 0.2	0.4 ± 0.1
% DHA of total FA:	11.4 ± 0.7		13.6 ± 0.3 #	↑	13.4 ± 0.5		13.7 ± 0.5		12.8 ± 1.1		12.6 ± 0.8		12.2 ± 0.8	13.6 ± 1.3 #
DHA conc; mg/g:	2.0 ± 0.5		2.6 ± 0.6		2.5 ± 0.2		2.5 ± 0.2		1.9 ± 0.5		1.8 ± 0.2		2.7 ± 0.3	2.9 ± 0.5
ω-6 / ω-3 ratio:	1.3 ± 0.1		1.0 ± 0.1 #	↓	1.2 ± 0.1		1.1 ± 0.1 #	↓	1.2 ± 0.1		1.1 ± 0.1 #	↓	1.3 ± 0.1	1.1 ± 0.1 #
ARA / DHA ratio:	1.0 ± 0.05		0.8 ± 0.1 #	↓	0.9 ± 0.05		0.8 ± 0.1 #	↓	0.9 ± 0.1		0.8 ± 0.1		1.0 ± 0.1	0.8 ± 0.1 #
** DHA concentrations are expressed as mg per g wet wt. of tissue. # p ≤ 0.05, for placebo vs. fish oil group, as by t-test.														
= Omega-6 polyunsaturated fatty acid = Omega-3 polyunsaturated fatty acid														

Figure 9. Table for the liver and brain fatty acid composition values of placebo versus fish oil treated rats, for shams and at 3, 7, and 28 days following double blast exposure (top and bottom panels; n = 5, 8, 8, & 8 and 6, 8, 8, & 8, respectively; mean ± SD). The individual omega-3 and omega-6 fatty acids (highlighted pink and light green, respectively) detected were C18:2 ω -6 (LA), C18:3 ω -6 (GLA), C18:3 ω -3 (ALA), C20:3 ω -6 (DHGLA), C20:4 ω -6 (ARA), C20:5 ω -3 (EPA), C22:4 ω -6 (DTA), C22:5 ω -6 (DPA), C22:5 ω -3 (DPA), and C22:6 ω -3 (DHA). ND = not detected, for brain ALA and EPA; since these two fatty acids are normally absent from the brain, due to β -oxidation processes. The most crucial fatty acids for our study are arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). General content of each fatty acid is expressed as a percentage of the total. Absolute concentration of DHA is expressed as mg per g wet weight of tissue. # p > 0.05; significant difference between dietary treatment groups, as by t-test. Blue shaded arrows indicate direction of each significant change.

VI. Cytokine Assessments of Brain and Plasma from Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

Frozen brain regions are partially thawed and then uniformly representative sections of cerebellum and mid brain are cut out with a scalpel blade. Total protein extracts (lysates) are made for the brain regions, by homogenization into a cell lysis buffer (T-PER; Thermo Fisher Scientific), followed by centrifugation to obtain the supernatant containing the cytokines. Frozen plasma is thawed and analyzed without additional processing. Samples are screened, using kits for immunoassay arrays (R&D Systems, Inc.), for simultaneous levels of 11 distinct cytokines, i.e., CXCL2, CXCL3, ICAM-1, IL-1- α , IL-4, IL-6, IL-10, IL-18, TIMP-1, TNF- α , and VEGF. These factors were chosen by us as a customized array for those that are already known to be upregulated during neuroinflammation. The array consists of a 96 well micro-titer plate based kit containing color-coded polystyrene nano-beads coated with analyte-specific capture antibodies, as well as biotin-streptavidin-PE conjugate detection antibodies. A dual-laser flow-based detector (Luminex technology) is used to precisely quantify the doubly tagged cytokines alongside 8 point standard curves made for each. Brain results are reported in pico-grams per milligrams of lysate total protein as determined by a colorimetric assay kit (Bio-Rad Corp) or for plasma given as in pico-grams per ml. When using assay kits from different lots, the manufacturer provided us with response adjustment factors for each cytokine, such that the resulting data sets could be reliably combined. These correction factors were also designed to bring

the array results into agreement with rat plasma values obtained for each cytokine using ultra-performance ELISA kits (i.e., single analyte) that they market. Thus, the brain and plasma cytokine concentration values that we are giving here can differ in magnitude from those cited in our previous reports. This, however, does not alter any of our previous conclusions that were entirely based on relative comparisons for individual cytokines between the dietary treatment groups at a specific end point.

Results and Conclusions:

Over the course of the entire study, we carried out cytokine assays for retinas and brains from a total of 40 placebo and 36 fish oil treated rats as shams and at 3 - 28 days post-blast. Likewise, corresponding plasma from these animals were analyzed for all rats, but only at 3 days out. Shown in Figure 10 are bar graphs for the retina, brain (two regions), and plasma (panels 1, 2, 3, and 4) cytokine levels of the placebo and fish oil treated rats as shams and at 3, 7, and 28 days following double blast exposure, (shams = green, PBO; placebo = red, and FO; fish oil = blue; n = 6, 10, 10 / 6, 8, 10 / 6, 11 & 10 [retina]; 11, 11, 10 / 11, 9, 10 / 11, 11 & 10 [mid-brain]; 15, 11, 10 / 15, 9, 10 / 15, 11 & 10 [cerebellum]; and 15, 11, & 10 [plasma], as grouped by treatment and day). To have enough assay material, the retinas from the right and left eyes were combined. We analyzed the cytokines found in the mid-brain, which contains the superior colliculus that is involved in visual processing. We also examined the cerebellum as a positive control; since it is known to be often severely injured in blasted animals. When we ran the assays, IL-10 in many samples was not reliably detected above background in the brain; and thus, it was dropped from the results and just 10 cytokines were reported. Likewise, for the plasma only CXCL3, ICAM, IL-18, and TIMP-1 showed levels detectable above background. To examine if any blast-injury effects were present in the retina, brain, and plasma from blasted animals, we did direct comparisons to the cytokine levels of sham placebo and fish oil treated rats that were lumped together to increase the statistical analysis power, since they did not show any apparent differences due to the diets alone. Due to the complexity of the results, the changes in each tissue at the end points will be separately addressed below without cross comparisons between them.

For the retinas (panel 1) at 3 days post-blast, ICAM-1 was significantly elevated compared to shams in both dietary treatment groups (1.4 and 1.5-fold). IL-18 and VEGF were significantly increased in the fish oil group alone (1.8 and 2.2-fold). Likewise, IL-1- α was significantly higher in the fish oil versus placebo rats (1.3-fold). Interestingly, TIMP-1 and TNF- α showed marked trend to be greater in both diet groups, but even more so in the fish oil animals. For the retinas at 7 days post-blast, ICAM-1 was only significantly increased versus shams in the fish oil group (1.3-fold) and IL-18 just for the placebo group (1.6-fold). IL-6, however, was significantly diminished in the fish oil treated rats versus shams and blasted placebo group (47 and 42%). The same trends for TNF- α and VEGF (i.e., large increases) at 3 days out were again observed at 7 days post-blast for both dietary treatment groups.

For the mid-brain (panel 2), which contains some of the visual processing centers, at 3 days post-blast TIMP-1 was significantly elevated above the shams, but only in the placebo group (1.9-fold). There were slight trends for both dietary treatments to be elevated in CXCL2, IL-6, and IL-18, and decreased in VEGF. The fish oil group alone, however, show a trend to be lowered especially in IL-1- α and TNF- α . For the mid-brain at 7 days post-blast, IL-18 was significantly higher in both dietary treatment groups compared to shams (1.3 and 1.5-fold); whereas, VEGF was markedly decreased for both diet groups (45 and 52%). There were modest trends for the fish oil group to be increased versus the shams in CXCL3, ICAM-1, and TIMP-1; and decreased in IL-1- α and TNF- α versus the placebo animals. For the mid-brain at 28 days post-blast, IL-18 and TNF- α were significantly higher in both dietary treatment groups compared to shams (4-fold, each and 5-fold, each); and, likewise, IL-1- α and VEGF were lower (32 & 35% and 23 & 29%). IL-6 and TIMP-1 were significantly elevated but only in the placebo animals (1.3 and 1.4-fold); whereas, CXCL3 was decreased (30%). There were modest trends for both diet groups compared to shams to be decreased in ICAM-1; and only the fish oil group in CXCL3. In contrast, the fish oil animals had a trend for increased IL-6 and TIMP-1.

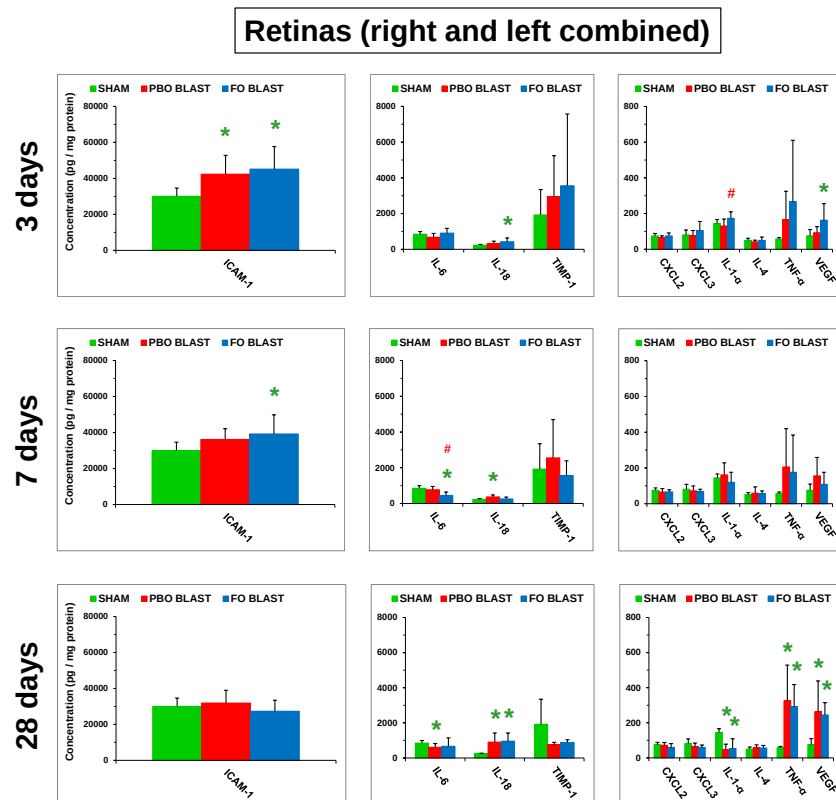
For the cerebellum (panel 3), which represents our blast-injury control, at 3 days post-blast CXCL2 and TIMP-1 were significantly elevated in both dietary treatment groups compared to shams (1.9 & 1.5-fold and 1.2 & 1.3-fold, respectively); whereas, IL-1- α and IL-4 were increased only in the placebo group (1.2-fold, each). There was a modest trend for IL-6 and TIMP-1 to be lower in the fish oil versus placebo treated rats. For the cerebellum at 7 days post-blast, IL-1- α was significantly higher than shams in both dietary treatment groups (1.5 and 1.7-fold); and CXCL3 and IL-4 were elevated but only in the fish oil animals (1.5 and 1.3-fold). There

were strong trends for both diet groups compared to shams to be decreased in VEGF; and the fish oil group to be increased in ICAM-1. For the cerebellum at 28 days post-blast, IL-18 and TNF- α were significantly increased in both dietary treatment groups versus shams (4-fold, both and 5 & 4-fold); in contrast, CXCL3 and ICAM-1 were decreased (30%, both and 24%, both). IL-1- α was significantly decreased, but only in the fish oil animals (34%); which were also lower in value than the placebo animals (24%). Likewise, IL-4 was elevated in the placebo group (1.3-fold). There was just a very marked trend for an increase of TIMP-1 in the fish oil group when compared to shams.

For the plasma (panel 4) at 3 days post-blast both TIMP-1 and IL-18 were significantly elevated in both dietary treatment groups to a nearly identical degree (1.5 and 1.7-fold). Likewise, CXCL3 showed a strong trend to be increased for both groups; and ICAM-1 was slightly lower in the fish oil treated rats. It is unknown, however, what alterations occur in these subtle differences in cytokines at 7 and 28 days post-blast.

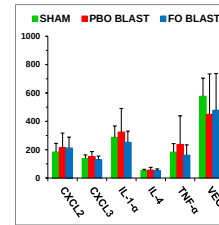
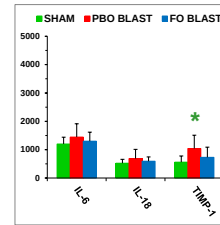
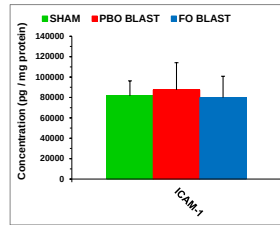
Overall, our findings imply that blast exposure produces marked changes in retina and brain cytokines that persisted out to 28 days post-blast, but the brain visual centers appear to be damaged to lesser degree than other more exposed regions (i.e., cerebellum). Also, it is apparent that plasma cytokine changes do not completely reflect those occurring in the retina and brain. In all four tissues, however, it was found that fish oil treatment at all times examined post-blast provided little if any advantage over the placebo group in suppressing levels of pro-inflammatory cytokines (e.g. IL-6) and increasing those known to help resolve inflammation (e.g. IL-18 and TIMP-1). Thus, it is possible that omega-3 fatty acids are relatively ineffective at modulating the release of these secondary messengers produced by activated immune cells or the beneficial effects lie at acute injury times earlier than 3 days post-blast, when cytokine responses have been reported by others to peak (i.e., within 24 hours).

Figure 10: Retinas, brain, and plasma cytokines of double blasted rats at 3 - 28 days post-exposure.

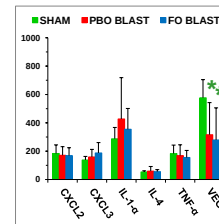
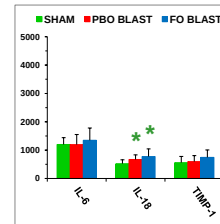
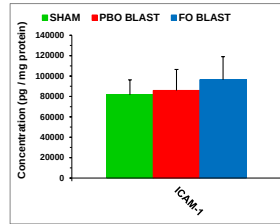


Mid-brain region

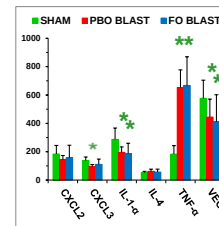
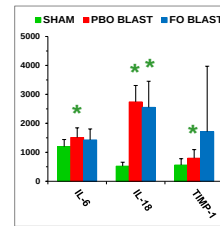
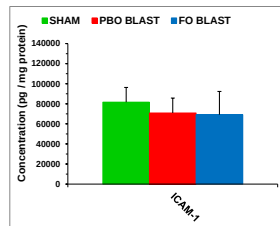
3 days



7 days

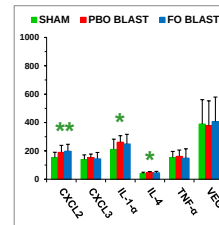
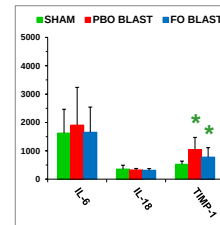
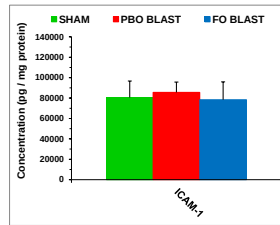


28 days

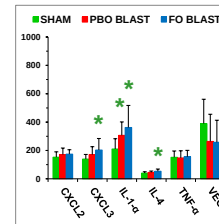
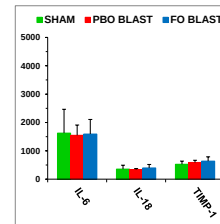
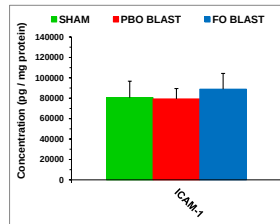


Cerebellum region

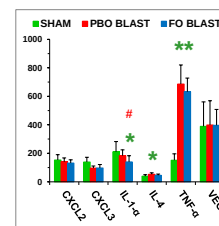
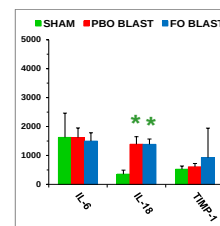
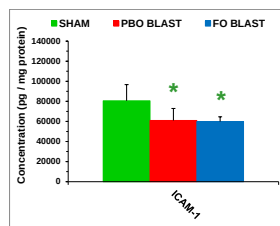
3 days



7 days



28 days



Plasma (3 days, only)

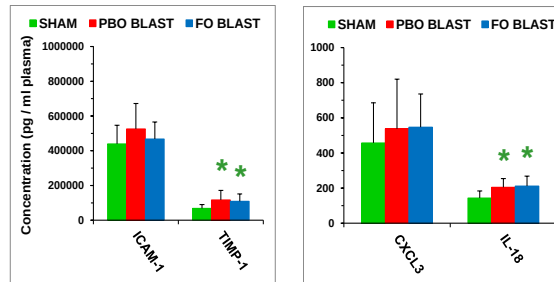


Figure 10. Bar graphs for the retina (first / top panel), mid-brain (second panel), cerebellum (third panel), and plasma (fourth / bottom panel) cytokine levels of placebo versus fish oil treated rats, as shams and at 3, 7, and 28 days following double blast exposure (shams = green, PBO; placebo = red, and FO; fish oil = blue; n = 6, 10, 10 / 6, 8, 10 / 6, 11 & 10 [retina]; 11, 11, 10 / 11, 9, 10 / 11, 11 & 10 [mid-brain]; 15, 11, 10 / 15, 9, 10 / 15, 11 & 10 [cerebellum]; and 15, 11, & 10 [plasma], as grouped by treatment and day; mean $\pm$ SD). Retinas were combined from right and left eyes. For each tissue, the data is broken into two rescaled frames to allow visualization of less abundant cytokines. Tissue concentrations (per mg total protein or ml plasma) for up to 10 cytokines are shown, i.e., CXCL2, CXCL3, ICAM-1, IL-1- α , IL-4, IL-6, IL-18, TIMP-1, TNF- α , and VEGF. . *p < 0.05; significant difference between shams and blasted rats, as by t-test. No significant differences were detected between the dietary treatment groups.

VII. Histopathology for Eyes and Brains of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

Rats from the placebo and fish oil dietary treatment groups will be euthanized at 3, 7, 14, and 28 days post-blast exposure for histopathology assessments of their eyes (retinas) and brain visual centers. Animals are deeply anesthetized by isoflurane inhalation. After surgical opening of the chest and insertion of gravity flow lines in the heart, the rats are perfused transcardially with physiological saline, which results in euthanasia by blood exsanguination, followed by phosphate buffered 4% paraformaldehyde saturated with picric acid (FD Neurotechnologies, Inc) to fix the tissues. Prior to saline perfusion, blood is taken by cardiac puncture via a syringe and placed in chilled EDTA and heparin vacuum collection tubes. Some blood will be centrifuged to obtain the plasma fraction, which is stored frozen at -80°C for future analysis. Whole blood and plasma are used for blood work and cytokine level assessments as previously described.

Eyes and brains are carefully removed from the paraformaldehyde perfused rat heads. To finish the fixation, brains are immersed for up to 6 hours in phosphate buffered 4% paraformaldehyde saturated with picric acid and then washed overnight with buffered 20% sucrose solution. To toughen, the eyes are post-fixed for 6 hours in a mixture of 2% trichloroacetic acid, 2% zinc chloride, 20% isopropanol as made up in paraformaldehyde without picric acid. Post-fixed eyes are washed with phosphate buffered saline followed by 50% ethanol, and then stored in 70% ethanol. Fixed eyes and brains are sectioned, stained, and mounted on microscope slides by FD Neurotechnologies, Inc. Brains are cut into serial coronal sections (30 - 50 μ m) through the cerebrum at 11 evenly-spaced positions from stereotaxic coordinates of bregma 1.0 mm to -8.3 mm, as mounted in triplicate. The brain sections target all major visual centers. Eyes are cut as a single horizontal section (5 μ m) passing through the central axis of the eye at the optic nerve, as done in triplicate.

Slides are prepared for the tissue sections that are stained with hematoxylin and eosin (H&E; eye and brain) and silver (brain only). H&E stain is reactive towards membrane lipids and proteins, and highlights the cytoplasm and nuclei of neurons as a pink to purple color. Silver stain is highly reactive to proteins, and highlights as a brown to black color the axonal fiber tracts of neurons. Both stains are able to reveal degenerating neurons by differences in morphology and staining intensity. Immunohistochemistry is done on

separate eye and brain sections (5 - 15 μm) for the presence of immune cells involved in inflammation that are well known to express the proteins Iba-1 (ionized calcium binding adaptor molecule 1), CD68 (cluster of differentiation 68), and GFAP (glial fibrillary acidic protein), i.e., activated microglia & macrophages, macrophages, and astrocytes, respectively. Sections are exposed to streptavidin-biotin conjugated primary antibodies toward these proteins (DAKO USA, AbD Serotec Bio-Rad, and Wako Chemicals, respectively), generating a dark brown coloration of cells overexpressing them, which have infiltrated and/or become activated within the neuronal tissues. Activation of microglia is also characterized by formation of ball and elongated shapes having diminished fiber extensions (non-ramified). For the eyes only the retinas are examined, whereas for the brain mainly the optic tracts were assessed, using an axial microscope having an image capture camera linked to a computer. Histopathology results are expressed as representative images, staining intensities, morphological perturbations, thickness areas, and/or cell counts. To carry out thickness area or immune cell count determinations on the retinas, 2 - 3 regions of interest (ROI) on each side of the optic nerve head (i.e., central, middle, and peripheral zones) were evaluated for three serial sections at 10 - 20x magnification. These ROI covered 60 - 80% of the total retina. For immune cell counts for the brain optic tracts, the entire structure on the section was outlined as the ROI. Typically, the best quality brain section out of three serial sections was used for the quantifications. Retina thickness areas or retina and brain immune cell counts were automatically tabulated using image analysis software (Image-Pro Plus, Media Cybernetics). Measurements were summed or averaged across the retina and brain optic tract ROI and reported for thickness areas as mm^2 and immune cell counts as $\text{cell}/\mu\text{m}^2$.

Results and Conclusions:

Over the course of the entire study, we carried out histopathology for eyes (retina) and brains from a total of 26 placebo and 27 fish oil treated rats as shams and 3 and 7 days following double blast exposure. While all of the eyes were assessed from these animals only 15 and 16 brains, respectively, were evaluated. We also did not carry out histopathology on eyes and brains from any blasted animals at 28 days out. For those done, tissue sections were processed into microscope slides for H&E and silver stains (eyes and brains, respectively) and CD68, GFAP, and Iba-1 immunohistochemistry (IHC; eyes and brains) and assessed by capturing representative images. Shown in Figure 11 are representative images for eye sections at 7 days post-blast (panels 1, 2, 3, and 4), which are close up shots (2 - 20x magnification) of the right side retinas from rats for the two dietary treatments. From immune cell counts as well as MRI data to be presented below, we know that 7 days out is well within the height of the inflammation response for the blast injured retina and brain. Under H&E stain, placebo and fish oil treated rats equally appear to have mild to moderate ongoing retinal degeneration, when compared to shams, which is consistent with the right eye having faced the oncoming blast wave (panels 1, 2, and 3). Left side retinas also had signs of retinal degeneration, but more often to a lesser degree (images not shown). In support of these results, Iba-1 and GFAP IHC for the right side retinas showed an extreme amount of activated microglia and astrocytes (e.g. Müller cells), respectively, to be present in both dietary treatment groups following blast exposure (panels 3 and 4). Location of the Iba-1 positive microglia within the retina was discerned by weakly counter staining the sections with cresyl violet, which accentuates the photoreceptor and bipolar cell layers (panel 4). Activated microglia cells are clustered near the ganglion cells (inner nuclear layer), which has been purposed by others to be most susceptible to damage by blast wave exposure. On the other hand, the activated astrocytes are most concentrated near the photoreceptors (inner plexiform layer), which is known to be the most sensitive part of the retina to physical disturbances, e.g., detachments. Also, we did preliminary CD68 IHC of the eyes, but found a complete absence of activated macrophages within the retinas of blasted rats; but instead were sporadically clustered within the choroid behind the retinal pigmented epithelium (images not shown), which implies immune cell infiltration into the eye's posterior chamber has not occurred by 7 days post-exposure.

Also, shown in Figure 11, are representative images of the brain sections (panels 5, 6 and 7) for corresponding rats from the two dietary treatments, which are close up shots (2 - 20x magnification) of the left side optic tract brain region. The left optic tract is innervated with the right retina through substantial axonal cross over (> 20%) at the optic chiasm. Under silver stain, both placebo and fish oil treated blasted rats appear to have ongoing axonal fiber tract degeneration in their left optic tracts, when compared to shams; however, it often appears to be more severe in the placebo group. The right side optic tracts also had signs of neurodegeneration by silver stain, but typically to a much lesser degree (images not shown). In support of these results, Iba-1, CD68, and GFAP IHC for the left optic tracts show robust levels of activated microglia, macrophages, and astrocytes respectively, to be present in both treatment groups following blast exposure

(panels 6 and 7). The density of these activated immune cells appears to be nearly equal in the two diet groups, except Iba-1 positive microglia maybe slightly lower in the optic tracts of the blasted fish oil treated rats. Overall, the brain and retina images on a gross scale agree that extensive neuronal degeneration is present post-blast and suggest that providing dietary omega-3 fatty acids fails to alleviate this.

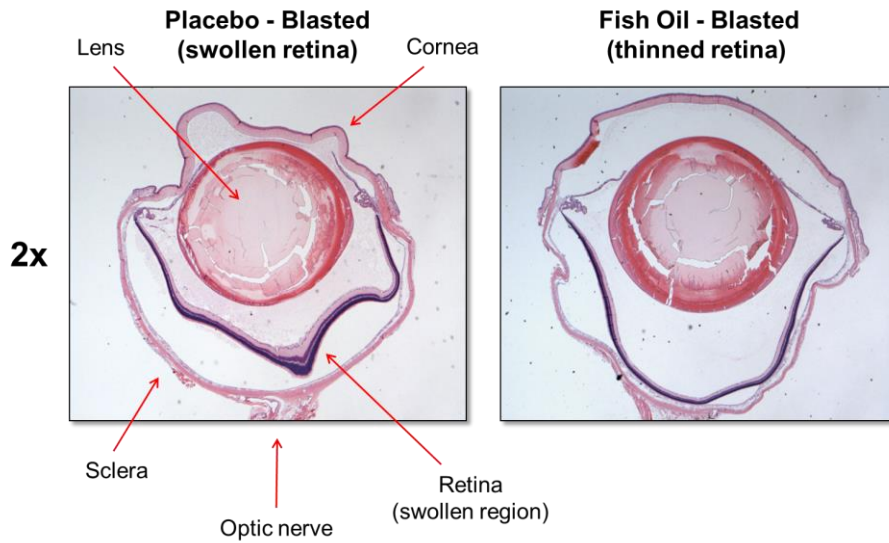
To more precisely determine if there is a lack of a treatment effect by omega-3 fatty acids on blast-induced neuroinflammation and neurodegeneration, we preformed thickness area measurements (retina) or immune cell counts (retina and brain) on the captured microscope images. Shown below in Figure 12 are bar graphs for the thickness total areas (mm^2) and activated microglia and reactive astrocyte densities (counts per μm^2) of left and right side retinas (panel 1; H&E stain, and panel 2; Iba-1 and GFAP IHC) and brain optic tracts (panel 3; Iba-1 and GFAP IHC) for placebo and fish oil treated rats as shams and at 3 and 7 days following double blast exposure (placebo = red and fish oil = blue; $n = 9, 12$ & 7 and $7, 12, \& 5$, respectively, for each). Also shown is a bar graph for the macrophage densities in the brain optic tracts at 7 days post-blast (panel 3; CD68 IHC); and in this case, the shams are combined single animals done for each diet (lumped shams = green, placebo = red, and fish oil = blue; $n = 2, 3$, and 3 , respectively). Interestingly, for the blasted eyes, we did not detect any significant differences in retinal thickness total areas between the placebo and fish oil groups. There was just a slight trend at 3 days out for the right retinas, which faced the blast, of both diet groups to be higher than their corresponding shams. This result was rather surprising, since we frequently observed the retinas of blasted rats to be grossly swollen (edema) and/or thinned (neurodegeneration) at the acute phase, i.e., by 7 days following exposure. It is possible that by averaging the thickness areas within or between retinas, these opposing changes balanced each other out. Thus, we will eventually pursue doing thickness area, as well as neuronal cell body count, measurements on specific retinal regions that appear to have the most blast-induced destruction (e.g. photoreceptors; inner plexiform layer).

Comparisons of activated immune cell densities in the retinas, however, showed marked inflammatory changes to be going on. Activated microglia (Iba-1 IHC) were found to be significantly higher in the left eyes of the blasted placebo group compared to the shams at the peak of 7 days out (2-fold); and had a marked trended to be elevated at this same time point in their right retinas. In contrast, the blasted fish oil group showed a slight trend to be higher at 7 days out versus the shams; and had a modest trend to be lower than the placebo group, suggesting that dietary omega-3s conferred a strong therapeutic benefit against this inflammation response. In contrast, reactive astrocytes (GFAP IHC) were significantly elevated above shams at 3 and 7 days out in the right and left retinas of both blasted diet groups (2 - 3-fold), or showed strong trends to be increased. Also, their astrocyte densities were relatively equal to each other, however, the fish oil group had a slight trend to be lower. This suggests that dietary omega-3 fatty acids have a limited ability, if any, to suppress the astrocytic response that is known to follow microglia activation.

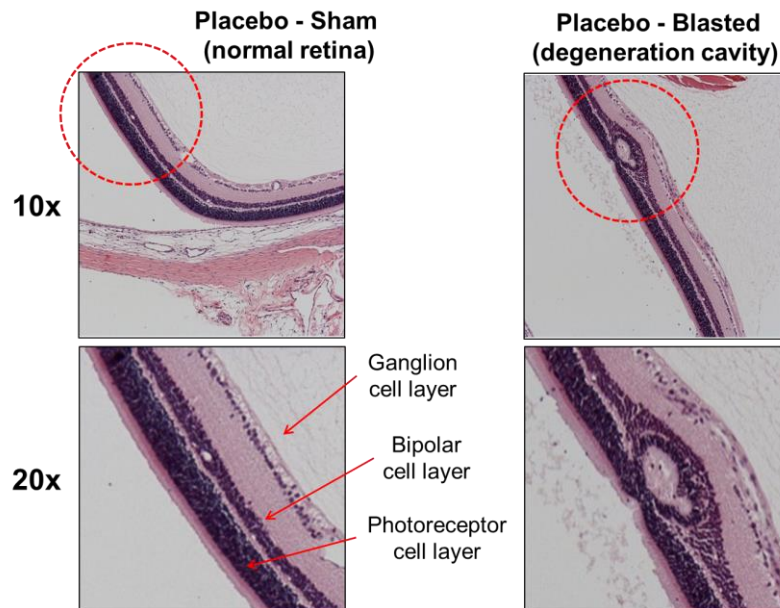
For the blasted brains, activated microglia (Iba-1 IHC) were significantly elevated in the right and left optic tracts of both dietary treatment groups versus shams at 3 and 7 days post-blast (2 - 3-fold); but trended to be even more so in the fish oil treated animals. The microglia changes were apparently more robust in the left optic tracts, which is consistent with their innervation with the right retina that faced the blast. Interestingly, the brain microglia activation showed a peak response at 3 days post-blast; whereas, in the retinas it was delayed until 7 days out. The early microglia response of the brain suggests that the optic tracts maybe more sensitive to blast damage compared to the retinas. Thus, some retina neuronal cell loss post-insult may arise from retrograde degeneration of the interconnecting axons that comprise the optic nerves, which is readily noted in most of the blasted brains by positive silver staining (Figure 11). Correspondingly, reactive astrocytes (GFAP IHC) were significantly increased in the both optic tracts to a similar degree with the peak at 3 days out (2-fold), but only in the blasted fish oil group compared to shams. The blasted placebo group had just a slight trend to be above the shams in reactive astrocyte counts, and to be substantially below the fish oil treated animals. Both diet groups were back down to normal reactive astrocyte levels at 7 days post-blast; although, the fish oil group lagged slightly behind for the left optic tract. To further confirm these results, we did a preliminary examination of the brain optic tract macrophage densities (CD68 IHC). For the left optic tracts of both dietary treatment groups the presence of macrophages was significantly increased to a tremendous degree compared to shams (80 and 60-fold); and the blasted fish oil animals had a strong trend to be less infiltrated with these immune cells. Macrophage counts for the blasted right optic tracts, however, showed only a moderate trend to be higher than the shams, with the two dietary groups being relatively equal. Overall, our findings suggest that dietary omega-3 fatty acids are again only able to slightly reduce immune cell guided neuroinflammatory responses following blast, especially in the brain visual centers.

Figure 11: Representative histopathology images for eyes (retinas) and brains of double blasted rats, at 7 days post-exposure.

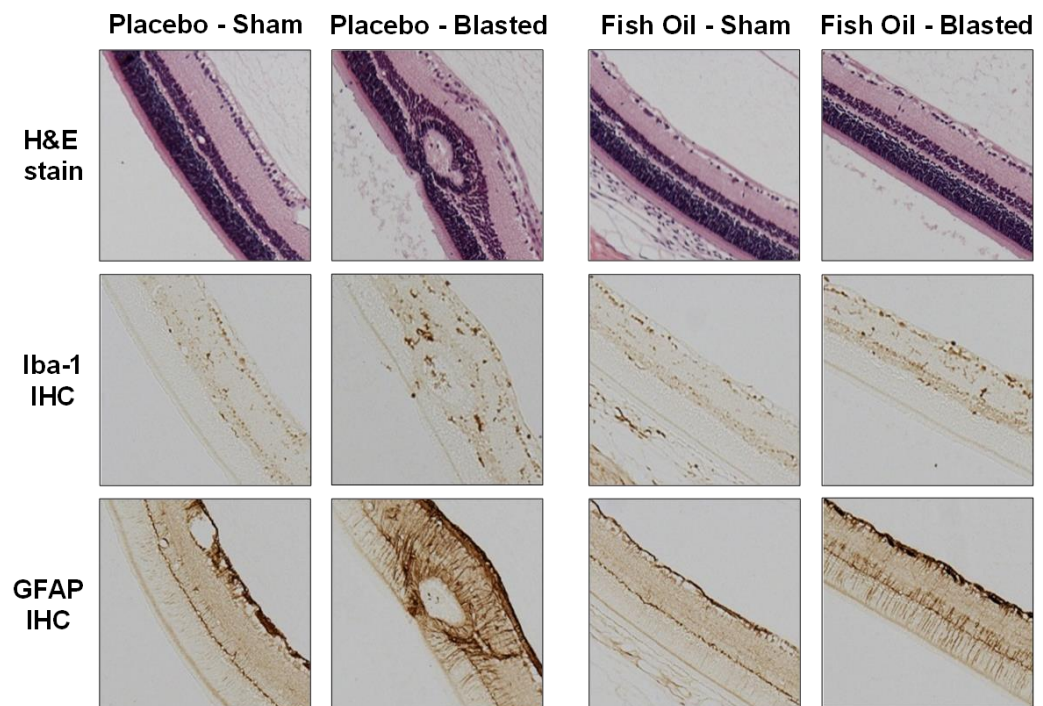
H&E stained blasted rat eye sections; position of the retina:



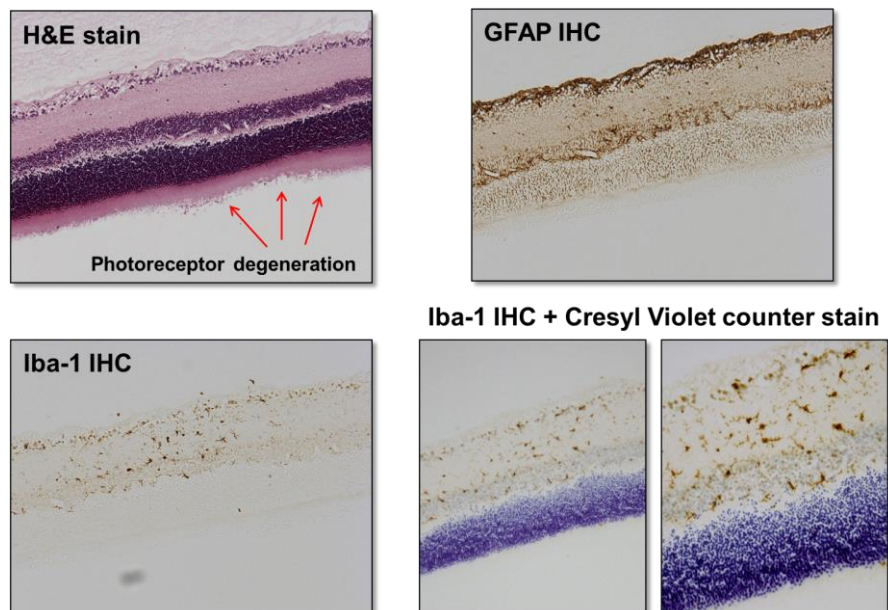
H&E staining of right side retinas for general cell morphology:



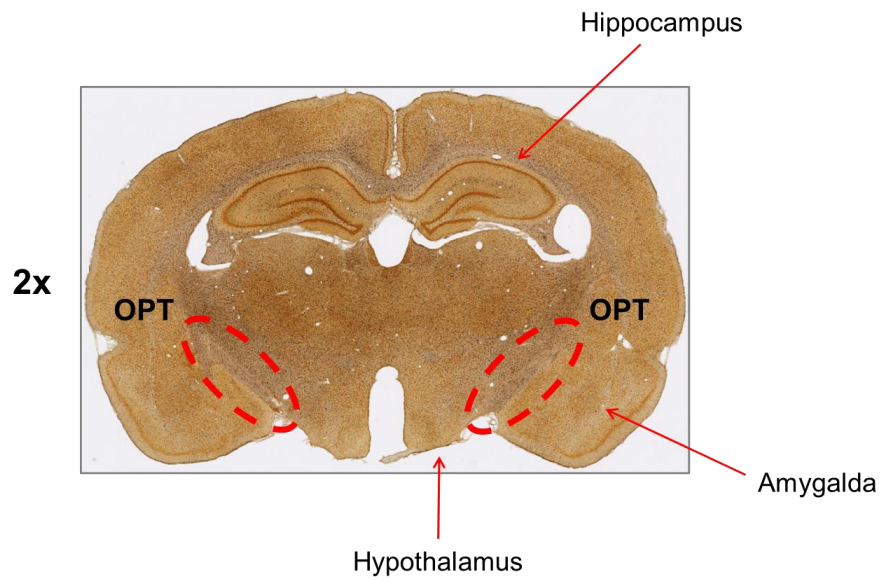
Iba-1 and GFAP IHC of retinas for activated microglia and astrocytes:



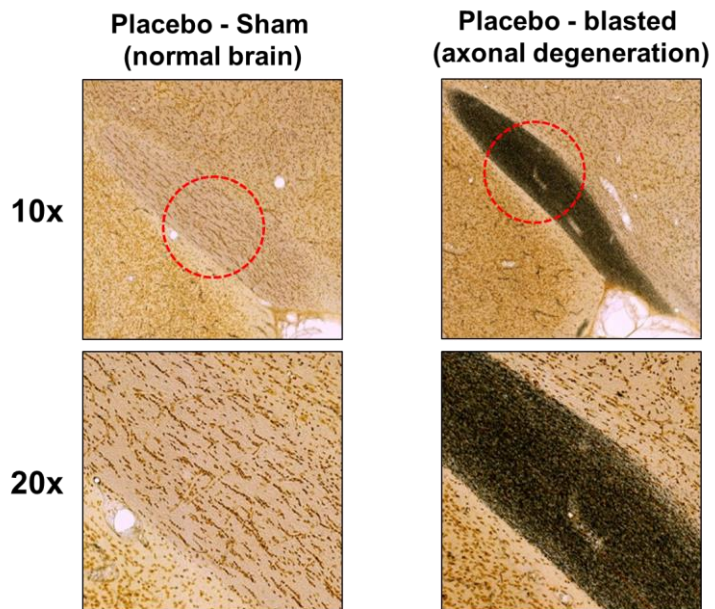
H&E, GFAP, and Iba-1 with cresyl violet counter stain for blasted fish oil treated retinas:



Silver stained fish oil sham rat brain section; position of optic tracts (OPT):



Silver staining of left side brain optic tracts for axonal fiber tract degeneration:



Iba-1, CD68, and GFAP IHC of brain optic tracts for activated microglia, macrophages and astrocytes:

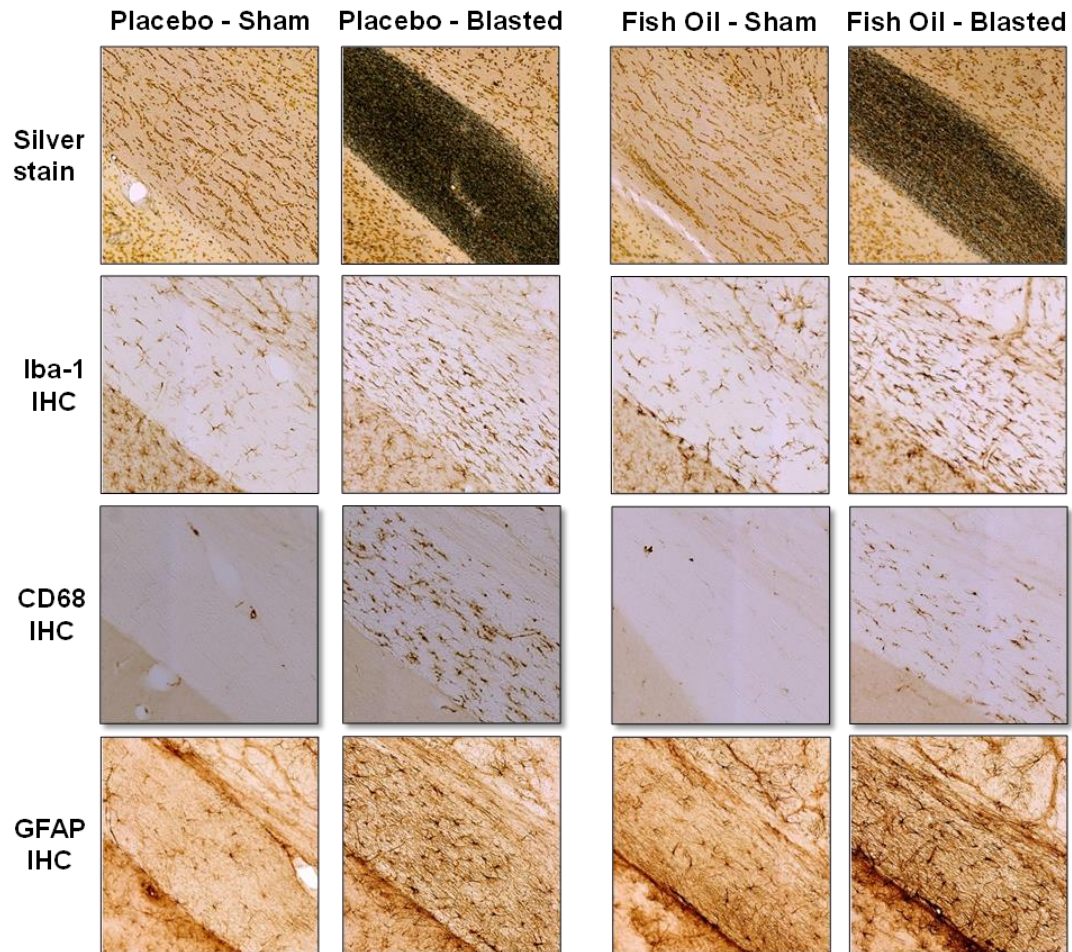
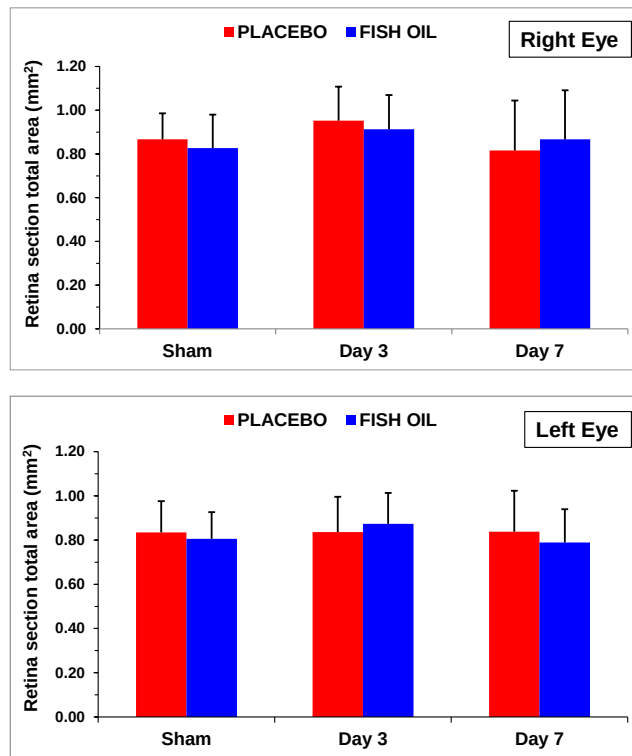


Figure 11. Representative images for eye sections (panels 1, 2, 3, and 4), which are close up shots (2 - 20x magnification) of the right side retinas from placebo and fish oil treated shams and double blasted rats at 7 days post-exposure. Likewise, representative images (2 - 20x) are displayed for the interconnecting left side brain optic tracts from the corresponding animals (panels 5, 6, and 7). Optic tracts lie directly between the hypothalamus and amygdala regions of the rat brain. H&E and silver stains highlight general cell morphology and axonal fiber tract degeneration, respectively. Iba-1, CD68, and GFAP IHC highlights activated microglia, macrophages, and reactive astrocytes, respectively. While Iba-1 IHC cross reacts to a small degree with macrophages, CD68 IHC is a specific towards these immune cells alone. Retina and brain optic tract images were taken at a higher power magnification to help reveal cellular feature

Figure 12: Histopathology for eyes (retinas) and brains of shams and double blasted rats at 3 - 7 days post-exposure; thickness areas and immune cell counts.

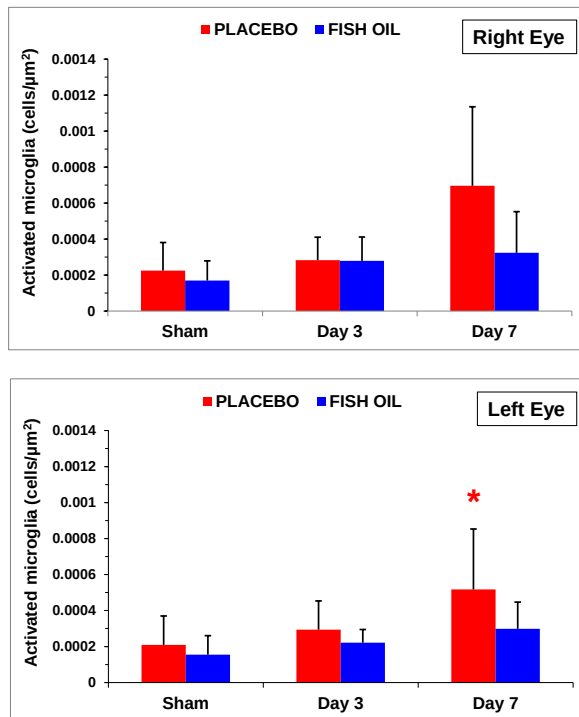
Retina histopathology, thickness area:

Retina section thickness total area; H&E stain

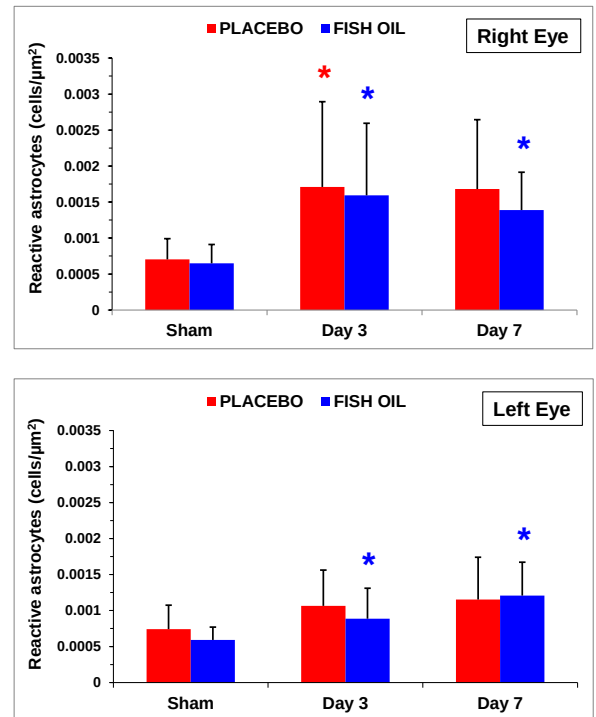


Retina immunohistochemistry, activated immune cells:

Retinas activated microglia ; Iba-1 IHC

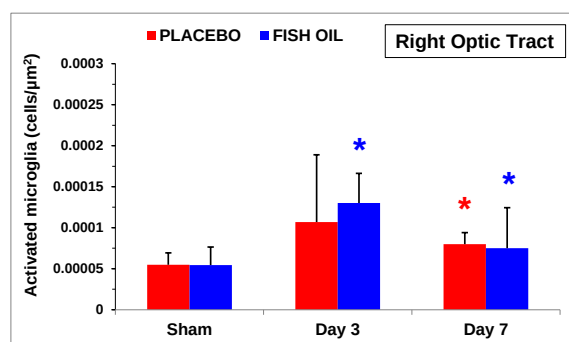


Retinas reactive astrocytes; GFAP IHC

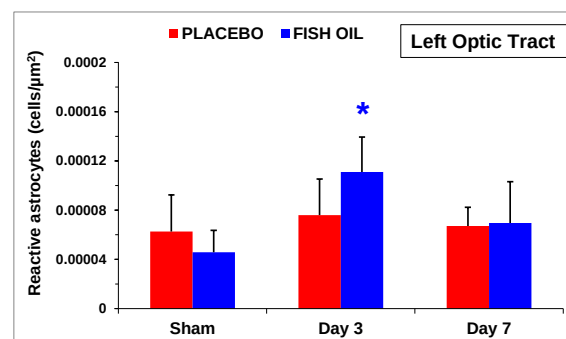
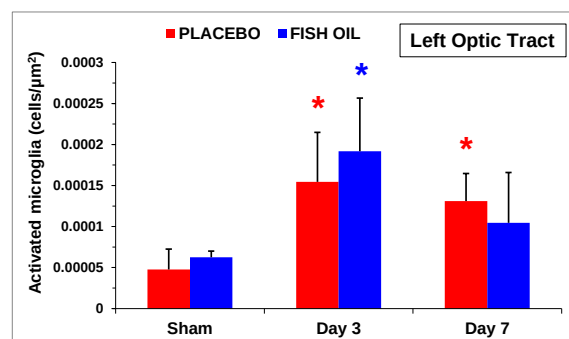
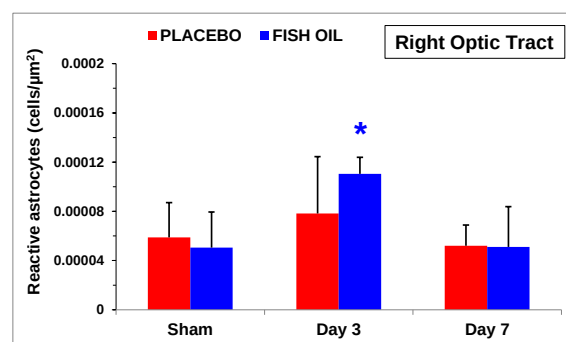


Brain optic tract immunohistochemistry, activated immune cells:

Brain activated microglia; Iba-1 IHC



Brain reactive astrocytes; GFAP IHC



Brain optic tract macrophages; CD68 IHC at 7 days out

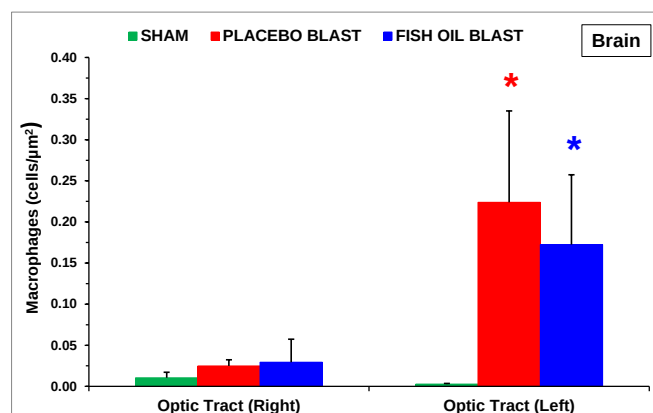


Figure 12. Bar graphs for the right and left retina and brain optic tract histopathology and immunohistochemistry assessments of placebo versus fish oil treated rats as shams and at 3 and 7 days following double blast exposure (placebo = red and fish oil = blue; $n = 9, 12$ & 7 and $7, 12$, & 5 , respectively, for each). Separate graph groupings are shown for retina morphology (panel 1; H&E stain), i.e., cross section total area (mm^2); and activated microglia and reactive astrocyte densities (counts per per μm^2) of retinas (panel 2; Iba-1 and GFAP IHC) and brain optic tracts (panel 3; Iba-1 and GFAP IHC). Also shown is a graph for brain macrophage densities (panel 3; CD68 IHC) of right and left optic tracts at 7 days post-blast (lumped shams = green, placebo blast = red, and fish oil blast = blue; $n = 2, 3$, and 3 , respectively). In this case, the results from single shams done for each dietary group were combined. * $p < 0.05$, significant difference from shams, as by t-test. No significant differences were detected between the dietary treatment groups.

VIII. MRI analysis for Eyes and Brains of Placebo and Fish Oil Treated Blasted-Rats.

Materials and Methods:

Twenty four hours preceding the experimental end points of 3, 7, and 28 days post-blast, rats receive intravenous injections of a commercially available perfluorocarbon (PFC) -based nanoemulsion, i.e., VSense-1000 or - 580H (Celsense, Pittsburgh, PA), for MRI Cell tracking of in-vivo labeled macrophages by ^{19}F MRI. The only difference between the agents is the perfluorocarbon molecule contained within the nanoemulsion; where the first is a perfluoro-crown ether and the second a perfluoro-polyether compound. Some animals alternatively receive a dual-mode imaging agent also containing a covalently-linked fluorescent dye (fluorescein; VSense-580H DM) to confirm cell/macrophage labeling by optical microscopy and histopathology. The PFC agent is non-toxic, readily taken up by macrophages as foreign particles, and the excess eliminated via hepatic and renal mechanisms. Fluorine-19 (^{19}F) is an abundant and stable (non-radioactive) isotope of fluorine that is NMR active (spin-1/2) nuclei with a gyromagnetic ratio similar to that of proton (^1H). An advantage to using PFC nanoemulsions for MRI cell tracking is that organic fluorine is not normally found in the body and ^{19}F MRI can unambiguously detect PFC-labeled cells in the absence of a background signal. Labeled cells are then placed in anatomical context with typical ^1H MRI.

Administration is done by placing the animals under isoflurane anesthesia; inserting a butterfly catheter with 25G needle into their lateral tail veins; and then by syringe pump, infusing the contrast agent at a saturating dose of ~ 3 ml/kg and rate of 0.5 ml/min. Circulating macrophages take up the reagent via endocytosis and home to sites of inflammation. At 24 hours, rats are anesthetized with isoflurane and perfused with saline followed by 4% paraformaldehyde saturated with picric acid. Fixed carcass are carefully sutured shut and sent out to our collaborators at the University of Pittsburgh's Animal Imaging Center (Dr. Hitchens' lab) for ^{19}F and ^1H -MRI analysis of the in situ eyes (retina) and brains. The PFC does not degrade the total ^{19}F found in a region of interest (ROI) provides measure of inflammation, i.e. an inflammatory index. Fixed rats were imaged using a small animal MRI scanner (Bruker, Biospin AVANCE III; 9.4 Tesla) equipped with a dual-tuned $^1\text{H}/^{19}\text{F}$ RF coil. Axial anatomical images were collected with a 2 mm slice thickness and 0.25 mm in-plane resolution. Following anatomical scans the RF coil is retuned from proton (400 MHz) to ^{19}F (377 MHz) scans repeated with signal averaging (NA = 64) and a 1 mm in-plane resolution for increased sensitivity to the dilute PFC label. Magnetic Resonance Microscopy (MRM) was also performed at 11.7 Tesla on the excised eyes and brains to more finely assess any apparent structural changes and/or macrophage deposits resulting from blast exposure. ^{19}F images are overlaid in "hot iron" pseudo-color onto corresponding ^1H images to place them into anatomical context and to assess ^{19}F -labeled macrophage accumulation for determining the distribution and severity of inflammation. Quantification of total ^{19}F -signals was performed for using a voxel tracking software (Voxel Tracker; Celsense). Regions of interest (ROI) were manually drawn around deposits of fluorine signals and assigned to a particular anatomy by consulting a rat anatomical atlas and anatomical MR images. Total fluorine signal for each ROI was summed and quantified using signal from a concentration reference standard in the imaging field of view. After imaging, the eyes and brains are shipped back to us for histopathology assessments, as previously described (Figure 11).

Results and Conclusions:

Over the course of the entire study, we submitted to the Animal Imaging Center at the University of Pittsburgh (McGowan Institute), for ^1H and ^{19}F -MRI analysis of the eyes (retina) and brains, a total of 35 placebo and 36 fish oil treated rats as shams or at 3 - 28 days post-blast. Image sets were returned to us for these animals and interpreted with help of the Animal Imaging Center staff. Shown in Figure 13 are representative structural anatomy (^1H -MRI) and corresponding macrophage cell tracking (^{19}F -MRI) images for the in situ & ex vivo eyes and brains (i.e., hippocampus, mid-brain, and cerebellum planes) of placebo and fish oil treated animals as shams and 3, 7, and 28 days post-blast (panels 1, 2, and 3). While neuronal tissue damage is not readily discernable on most of the structural anatomy views at the scanning resolutions used, the overlays and side by side comparisons with the corresponding ^{19}F -MRI scans show marked deposits of PFC-labeled macrophages, as displayed in "hot iron" pseudo-color, are occurring at potential blast induced inflammation sites in both dietary treatment groups compared to the shams. For the eyes in the full head / in situ and isolated tissue / ex vivo scans (panels 1 and 2), the blasted rats show extensive macrophage infiltration to be present in the raw images, which fills the entire eye globe space and shows only a slight preference for the

right versus left eye, despite the blast wave impact being focused at the right side of the head. The peak of the inflammation response appears to be at 3 - 7 days post-blast and may persist to a small degree out to 28 days. The shams, however, also show the presence of substantial residual ^{19}F -signal, especially for the eyes, which likely represents the natural population of macrophages found within this tissue. Likewise, macrophage accumulation appears to be relatively the same in the blasted diet groups at all end points, for both their eyes and brains. Interestingly, on the whole head images (panel 1) there appears to be also extensive macrophage accretion (i.e., inflammation) in the nares of the rat's nasal cavity, which maybe slightly less in the fish oil treated animals. Also, shown in Figure 13 are images of the corresponding brains, i.e., mid-brain and cerebellum (panel 3, top) for the rats from the blasted dietary treatment groups. As for the eyes, these brain regions in the blasted rats, as compared to shams, show substantial macrophage infiltration that primarily lies between the meninges and skull surrounding the mid brain and cerebellum, with apparent lack of penetration into the cortical tissue or ventricles. This suggests that the blood brain barrier (BBB) is no longer disrupted from the blast and the macrophages cannot readily pass through or the MRI contrast agent was unable to effectively get across the BBB to target macrophages already inside the brain. Again, from these raw images there is no strong indication that brain macrophage accretion is reduced in the fish oil versus placebo group. The brain images also suggest that macrophages are heavily aggregating near the bottom of the ear canals, likely within the inner ear chamber (panel 3, top, bold yellow arrow), which similar to our findings for the nasal cavity, and would suggest that the blast wave is being transmitted down these channels into the skull and then brain.

Of great noteworthiness, the MRI analysis picked up a macrophage filled lesion deep within the brain of one of the blasted fish oil treated animals, which may be due to ongoing BBB disruption in this subject. As also shown in Figure 13, are the overlaid ^1H - and ^{19}F -MRI brain scans for this individual animal (panel 3, bottom) of three coronal planes passing through the striatum to ventral hippocampus regions. In these images, there is a small "hot" cavity (i.e., macrophage filled) visible that is located on the right side of the brain centered within the somatosensory / auditory cortex and adjacent to the thalamus (yellow arrows). Directly ipsilateral to this, on the ^1H -MRI scans (i.e., structural anatomy), is an apparent contusion buried deep inside the left cortex (turquoise arrows). This could represent a counter coup strike of the brain against the skull; or as its distinctly round shape implies, a cavitation bubble produced by the shock wave propagating through the right brain and exiting the opposite side. Indeed, other brain regions discernable in these images show similar tissue distortions, e.g. the top of the thalamus. This is an exciting result, since it has been hypothesized by others that blast waves can cause destructive cavitation events within the brain; although, thought to be on a low micro-scale order. Histopathology assessments, however, are required to confirm the nature of the apparent blast induced neuronal injuries at this site.

For the MRI assessments, it is essential that we provide some corroborative evidence that the ^{19}F -signal is directly representative of macrophages present within the tissues. We had originally planned to do this by using histopathology performed on rats given fluorescein dye tagged contrast agent and looking for lit up macrophages in specially prepared eye and brain sections; however, we have not completed this task as yet. As shown in Figure 14, however, we noted that one blasted placebo rat in particular had an extremely robust ^{19}F -signal on MRI (panel 1, left) that emanated from only the right eye (yellow arrow), which faced the blast. This represented a unique preliminary opportunity to verify via standard histopathology that the ^{19}F -MRI results correspond to an increased presence of macrophages. Indeed, H&E stain and Iba-1 IHC based histopathology of the same right eye, showed the entire field of the retina (panels 1 and 2; rows 1 and 2) to be in a state of severe neurodegeneration with macrophages packing the tissue and nearby intraocular space. Moreover, GFAP IHC (panel 2; row 3) of the retina also showed it to be crowded throughout all neuronal cell layers with reactive astrocytes. In contrast, the left eye histopathology (panel 1; GFAP IHC not shown) was clean of these disturbances; thus, helping to validate our ^{19}F -MRI method.

Shown in Figure 15 are bar graphs for quantification of the total ^{19}F -signal (macrophage deposits) in the right and left eyes of placebo and fish oil treated rats as shams and at 3, 7, and 28 days following double blast exposure (placebo = red and fish oil = blue, $n = 8, 11, 8, \& 8$ and $8, 12, 8$ and 8 , respectively). Also, included are graphs for the ^{19}F -signals over time post-blast of the major bones comprising the surrounding eye socket (orbit), i.e., maxilla, zygomatic, frontal, and sphenoid, which are illustrated in the provided rat skull diagram (panel 1, top). MRI analysis of the in situ eyes (panel 1; row 1) showed that there was significant accumulation of macrophages in the right eyes of the blasted placebo group compared to shams at 7 days out (2-fold); and these animals also had a strong trend to be higher than the shams in the right eyes at 3 and

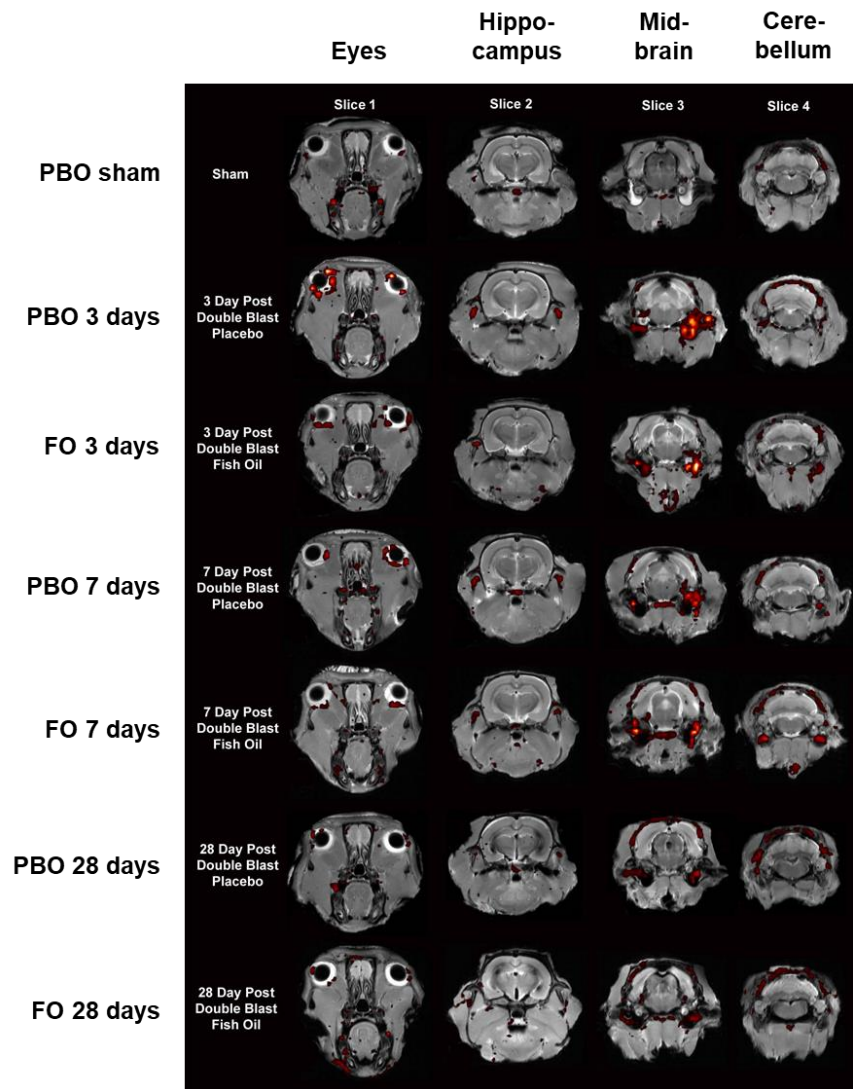
28 days out and the left eyes at 3 - 28 days out. In contrast, the blasted fish oil treated animals had ^{19}F -signals for the in situ eyes that were similar to or below the shams at all days out examined. Corresponding ^{19}F -signals of the orbital bones (panel 1; rows 2 - 4), showed that the blasted placebo group versus shams had significant increases in macrophage accumulation for the right maxilla (7 days out; 2-fold), right zygomatic (28 days out; 2-fold), and right frontal (7 and 28 days out; 3 and 4-fold) regions; and in particular there was a very large trend for the left zygomatic bone of these animals to be increased in macrophage content. The sphenoid bone lining the back of the eye, however, was apparently unaffected by the blast. Similarly, the blasted fish oil treated animals were significantly elevated above the shams for the right and left maxilla (7, 28, and 7 days out; 2-fold each), left zygomatic (7 days out; 3 fold), and frontal (7 days out; 3-fold) bones. In contrast, these animals had a significantly increased macrophage infiltration in the sphenoid bone at 28 days out (3-fold). There was also a strong trend for the blasted fish oil animals compared to shams to have higher ^{19}F -signals for the left zygomatic (3 and 28 days out), frontal (7 days out), and sphenoid (3 and 7 days out) bones. We did not, however, detect any significant differences between the two dietary treatment groups for the in situ eyes or facial bones.

As also shown in Figure 15 (panel 2), the eyes from the shams and blasted diet groups were fully excised and analyzed for their total ^{19}F -signals, using higher resolution MRI scanning (i.e., 11.7 Tesla). The macrophage patterns observed for the excised eyes were similar to that of above; but was partially reversed, with instead the right eyes of the blasted placebo group displaying the highest trend at 3 days out to be increased above the shams; whereas, the blasted fish oil animals were equally increased in both eyes. The switch to a higher ^{19}F -signal in the blasted right eyes, when imaged isolated as opposed to in situ, maybe due to the removal of damaged tissue surrounding the left eye (e.g. adipose and muscle) that had accumulated macrophages. Regardless, as before, the macrophage infiltration was more robust in the blasted placebo group. Indeed, for the right eyes at 7 days post-blast, the fish oil treated rats were significantly decreased in ^{19}F -signal compared to the placebo group (61%); and this difference was also present as a strong trend at 3 and 28 days out.

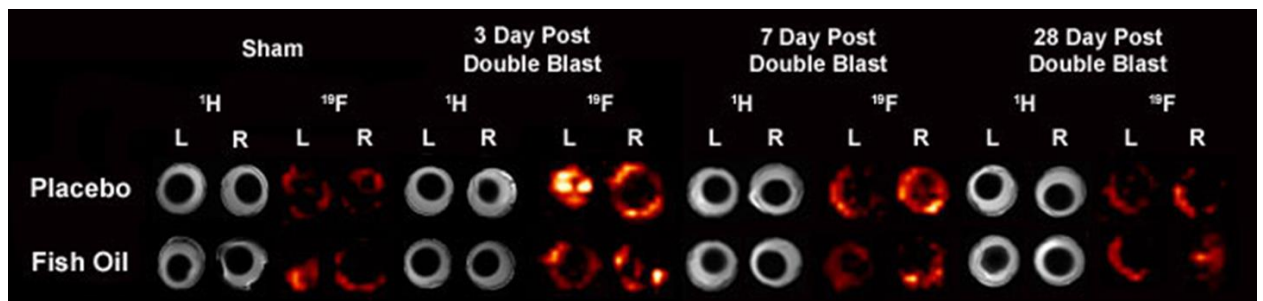
Overall, our MRI findings suggest that there is marked accumulation of macrophages in both eyes of the blasted placebo treated animals compared to shams and the blasted fish oil group. Interestingly, the inflammation was found to impact the left eye region somewhat greater, especially for the in situ imaging, despite the right eye facing the oncoming blast wave. This could be due to the blast wave wrapping around the head, transitioning through the skull, and/or reflecting off of the tube walls and striking the left eye, in an amplified state. Interestingly, corresponding ^{19}F -signals of the major bones making up the orbital socket suggested that especially the zygomatic bone, a thin arch that sits at the bottom-front of the eye, is severely inflamed in the blasted placebo rats. This implies facial bone fractures are occurring during the blast, which could exacerbate the eye injuries. Our MRI results showing some reduced accumulation of macrophages in the eyes of the blasted fish oil rats are very supportive of dietary omega-3 fatty acids as having a modest efficacy towards alleviating blast wave induced visual system injuries, which is in good agreement with our other outcome measures, i.e., ERG and retina histopathology.

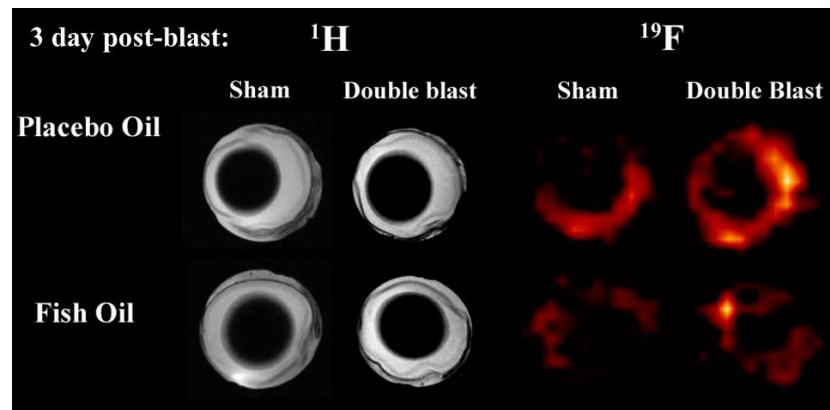
Figure 13: Representative MRI scans for eyes and brains of double blasted rats at 3 - 28 days out.

MRI (^1H and ^{19}F) based structural anatomy and macrophage cell tracking; head scans:

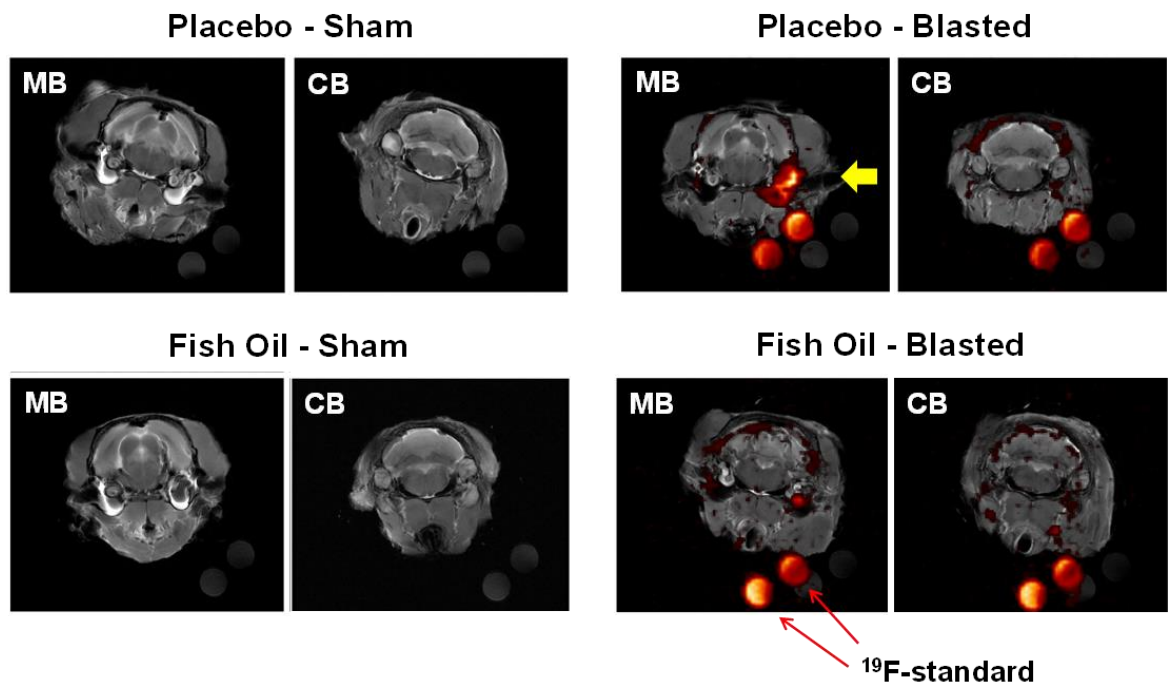


MRI based structural anatomy and macrophage cell tracking of excised eyes:





MRI based structural anatomy and macrophage cell tracking of brains at 3 days post-exposure:



Fish oil - blasted: striatum to ventral hippocampus scans (excised brain)

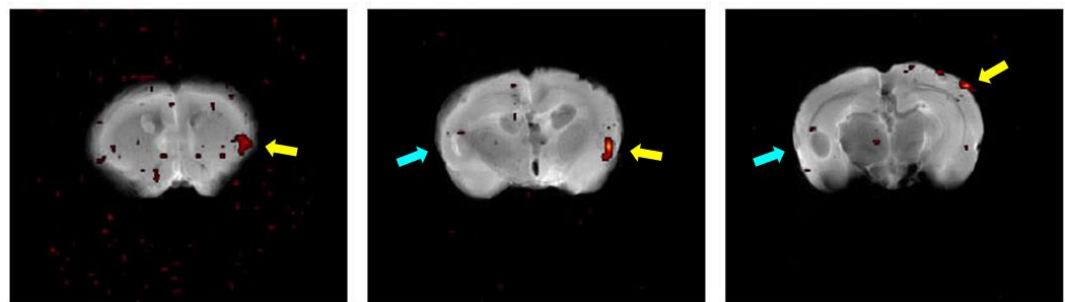
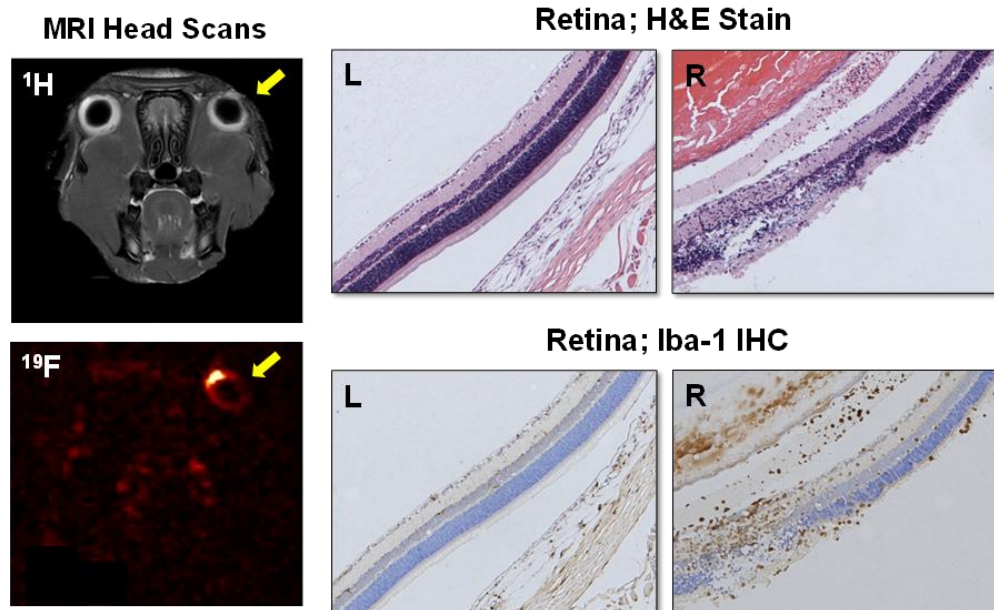


Figure 13. Representative structural anatomy (^1H -MRI) and the corresponding macrophage cell tracking (^{19}F -MRI) images for the heads at the level of the eyes and brain, i.e., hippocampus, mid-brain, and cerebellum planes, of placebo (PBO) and fish oil (FO) treated rats, as sham and 3, 7, and 28 days following double blast exposure (panel 1). Also, shown are enlarged images of the excised eyes (panel 2) and in situ & excised brains (panel 3). The ^{19}F -signal for all images is displayed in “hot iron” pseudo-color, which is an indicator of macrophage infiltration. The ^1H - and ^{19}F -signals are shown as superimposed for full head scans (i.e., in situ eyes and brains) and excised brain, and as separately for the excised eyes. As found on some brain images (panel 3, top), a ^{19}F -standard was applied next to the tissue for quantification purposes. The bold yellow arrow for the enlarged image of the mid-brain for a blasted placebo treated rat (panel 3, top) points to macrophage deposits likely located at the bottom of the ear canal, i.e., the inner ear chamber. MRI scans of an excised brain of a blasted fish oil treated rat are also shown (panel 3, bottom), as captured at coronal planes passing through the striatum to ventral hippocampus. Brain contusion and contralateral macrophage infiltration sites are indicated on these images (turquoise and yellow arrows, respectively). All ^1H -images and resulting ^{19}F -signals were determined first with the in situ eyes and brains at 7.0 Tesla and then with the excised tissues at 11.7 Tesla.

Figure 14: MRI and histopathology for eyes of a double blasted placebo rat at 3 days post-exposure.

MRI head scans and eye (retina) histopathology; H&E stain and Iba-1 IHC:



Right retina histopathology expanded view; H&E stain and Iba-1 IHC:

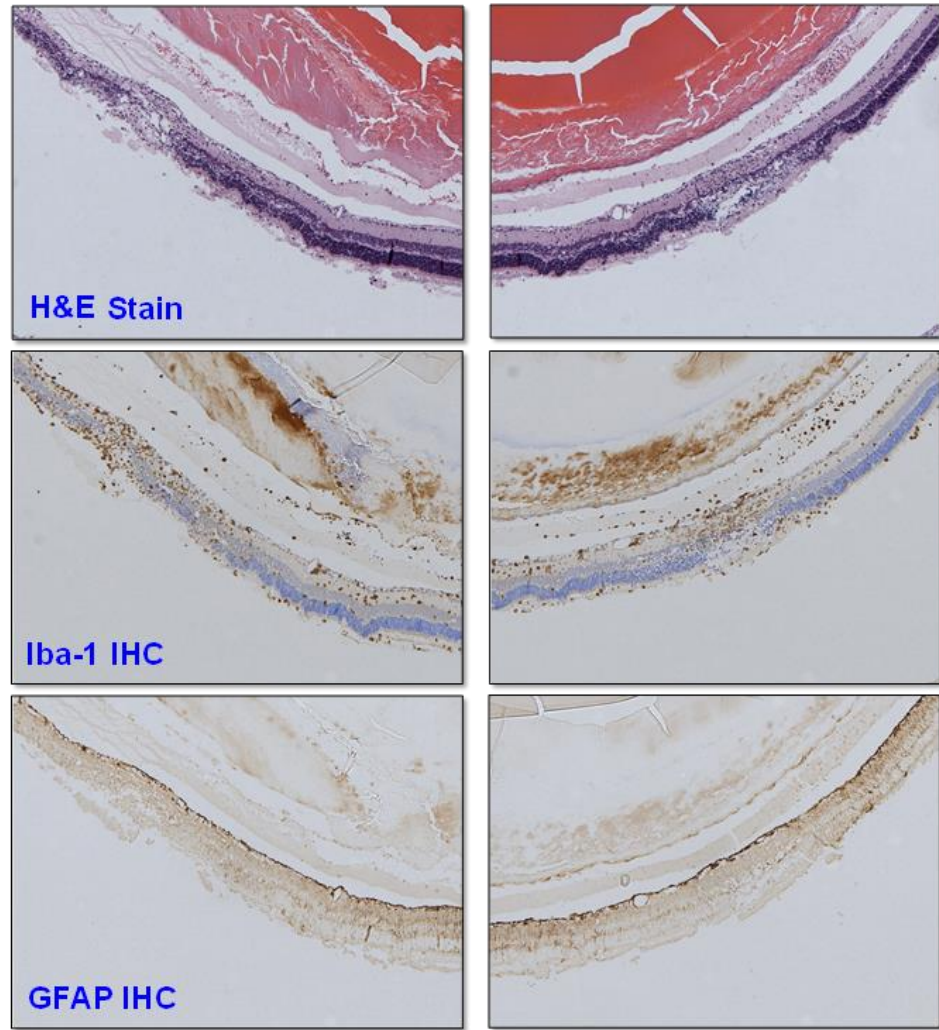
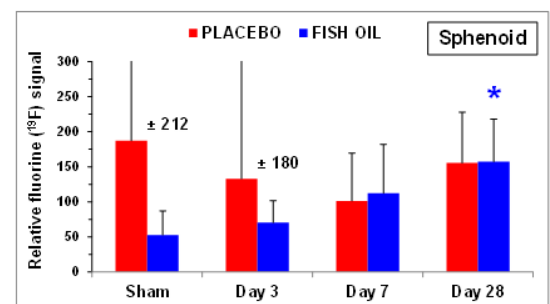
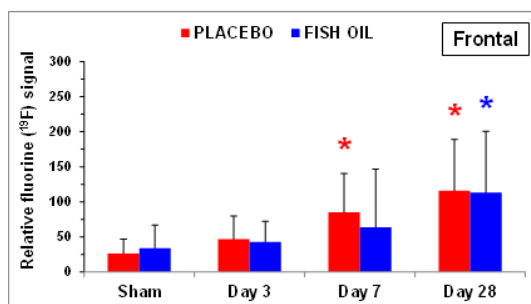
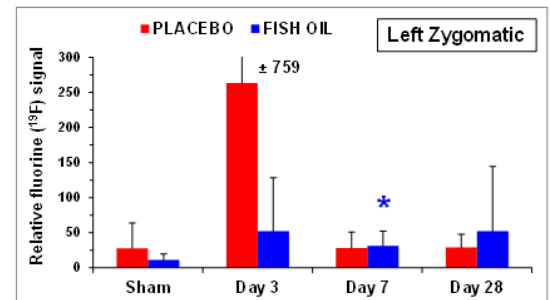
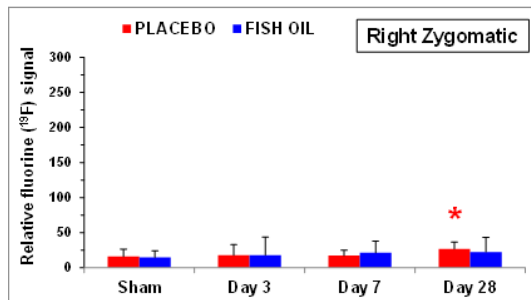
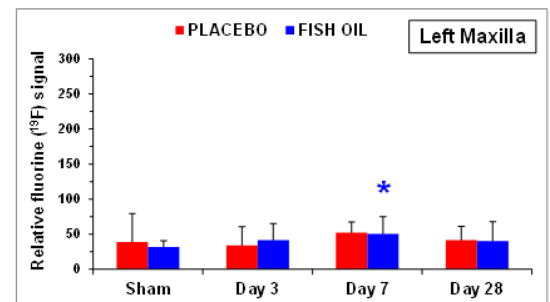
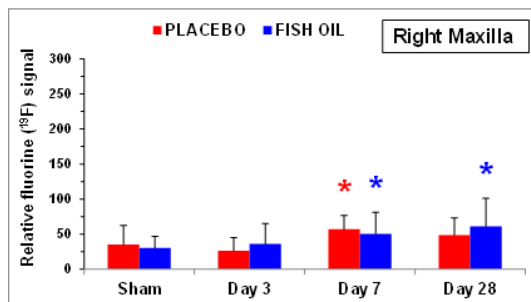
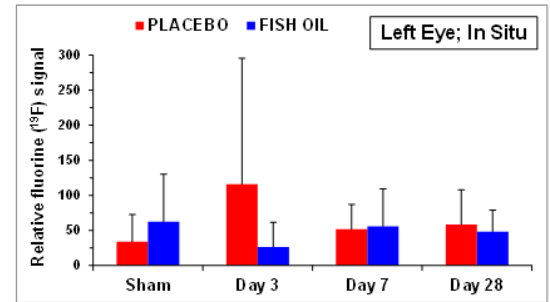
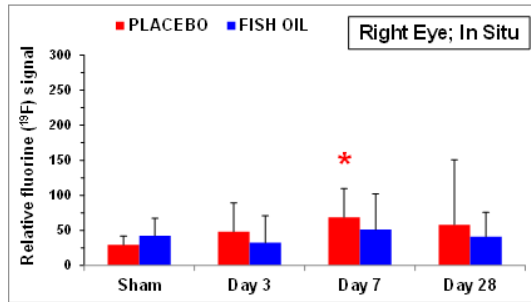
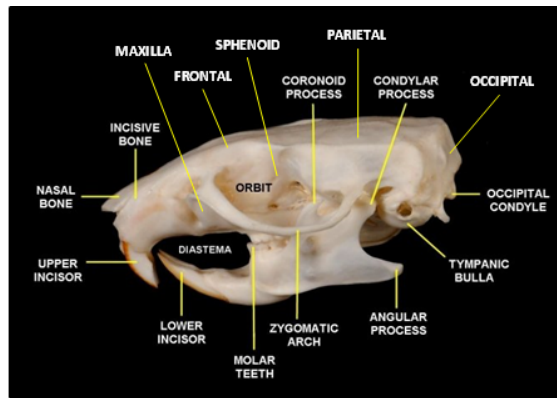


Figure 14. MRI and histopathology of a representative placebo oil treated rat at 3 days post-blast. In situ ^1H and ^{19}F -MRI (anatomical and macrophage images) of the rat's head is shown at a coronal plane passing through both eyes (panel 1). Retina histopathology for the corresponding left and right (yellow arrow) eyes is shown for H&E stain for degenerating neurons (e.g. photoreceptors) and Iba-1 IHC for microglia and macrophages, as taken at 10x magnification. Below this (panel 2) are wide field views (stitched; 4x magnification) of the right retina that took the brunt of the blast wave insult, i.e., H&E stain, and Iba-1 as well as GFAP IHC for activated astrocytes.

Figure 15: ^{19}F -MRI signal assessment of double blasted rats at 3 - 28 days post-exposure.

Macrophage infiltration status (total ^{19}F -signal) of in situ eyes and orbital bones:



Macrophage infiltration status (^{19}F -signal) of the excised eyes:

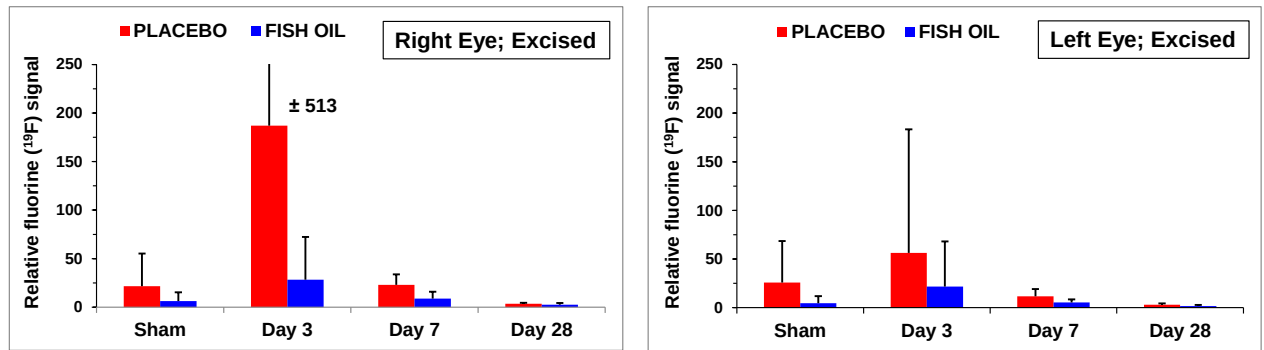


Figure 15. Bar graphs for the ^{19}F -MRI signals, as an indication of macrophage infiltration density, found in the right and left eyes of placebo versus fish oil treated shams and double blasted rats at 3, 7, and 28 days post-exposure (placebo = red and fish oil = blue, $n = 8, 11, 8, \& 8$ and $8, 12, 8$ and 8 , respectively; mean $\pm$ SD). Total ^{19}F -signals, on a relative scale, are shown for in situ eyes and primary facial bones comprising the surrounding orbital socket, i.e., maxilla, zygomatic, frontal, and sphenoid (panel 1), as carried out at 7.0 Tesla. The ^{19}F -signals for the excised eyes are also shown (panel 2), using high resolution scanning done at 11.7 Tesla. Y-axis for all graphs can be directly translated into ^{19}F spins $\times 10^{20}$, as detected by the MRI probe. * $p < 0.05$, significant difference from shams, as by t-test. # $p < 0.05$, significant difference between dietary treatment groups, as by t-test.

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

- 1) Attended the National Capitol Area TBI symposium held at the NIH in Bethesda MD, on 04 - 05 April 2016. At the meeting, there were many poster presentations on clinical aspects of visual system injuries in US soldiers after blast wave exposure. I was able to touch base with our local colleagues working in the field of blast induced neurotrauma. I also presented a poster on my initial data from the project at this meeting.
- 2) Attended the National Neurotrauma Society Symposium held in Lexington KY, on 25 - 30 June 2016. At the meeting there were many talks and poster presentations on treatment of neuronal damage to the retina and brain due to various forms physical injury. Most importantly, I was able to connect with Dr. Tonia S. Rex, from the Vanderbilt University Eye Institute - who models air blast induced ocular injuries in mice, and verify the validity of my retina injury findings with her. I also made connections with Dr. Andrew J. Morris, from the Biomedical Mass Spectrometry Core Laboratory at the University of Kentucky, who specializes in detection of trace lipids in tissues. He is willing to screen (without charge) my rat retina and brain samples for changes in levels of anti-inflammatory metabolites of omega-3 fatty acids, e.g. neuroprotectins. I attended several workshops focusing on animal models of neurotrauma, neuroprotective drug discovery, and brain histopathology techniques. I also presented a poster on my updated data from the project at this meeting.
- 3) Attended the Military Health Systems Research Symposium (MHSRS) held in Kissimmee / Orlando, FL, on 15 - 18, August 2016. At the meeting, there were many talks and poster presentations on aspects of traumatic brain injuries, including those to the visual system, in animal models and US soldiers after blast wave exposure. The focus of this meeting was broad, covering blast physics, injury pathologies / diagnosis, and prevention / treatment strategies. I was able to touch base with our local, national, and international colleagues in the field of blast induced neurotrauma. I also presented a poster on my updated data from the project at this meeting.

4) Attended the National Capitol Area TBI symposium held at the NIH in Bethesda MD, on 08 - 09 March 2017. At the meeting, there were many poster presentations on clinical aspects of visual system injuries in US soldiers after blast wave exposure. Most importantly, I made contact with a local investigator, Dr. Ji Hyun Lee, who is at the Translational Imaging Facility, Department of Radiology and Radiological Sciences, Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD. She was interested in establishing future collaborations with our lab to do advanced MRI, PET, and soft X-ray imaging on our blast wave exposed rats. Towards that effort, we toured their imaging facility on 18 April 2017. I also presented a poster specifically on my MRI and histopathology data from the project at the NCA-TBI meeting.

5) Our collaborators at the University of Pittsburgh (Dr. Hitchens' lab) presented an electronic poster for the updated MRI and histopathology findings of the project at the International Society for Magnetic Resonance in Medicine (ISMRM) 27th Annual Meeting held in Honolulu HA, on 22 - 27 April 2017. While I did not personally attend this meeting, Dr. Hitchens was able to provide me with contact information for other MRI labs that are interested in collaborating with us on rat imaging based blast projects.

6) Attended the Arrow Head Traumatic Brain Injury 7th Annual Conference held in Washington DC (Arlington, VA), on 24 - 25 May 2017. At the meeting there were many talks on advances in diagnosis and treatment of traumatic brain injuries, at both the civilian and military level. Most importantly, I made a connection with a biotech company, Ischemix Inc. (North Grafton, MA), which was looking for a laboratory to test their unique anti-oxidative stress drug (CMX-2043; α -lipoic acid analogue) in an advanced traumatic brain injury model related to military medicine. I am currently pursuing an MTA or CRADA with them, to do a pilot study of their drug in blast wave exposed rats to first see if it effectively crosses into the eye (retina) and brain, as well as knocks down acute biomarkers of neuroinflammation. I also presented a poster on my updated MRI and histopathology data from the project at this meeting.

7) Attended the Military Health Systems Research Symposium (MHSRS) held in Kissimmee / Orlando FL, on 27 - 30 August 2017. At the meeting, there were many talks and poster presentations on aspects of traumatic brain injuries, including those to the visual system, in animal models and US soldiers after blast wave exposure. The focus of this meeting was broad, covering blast physics, injury pathologies / diagnosis, and prevention / treatment strategies. I was able to touch base on my findings with the director of the University of Pittsburgh's Safar Center for Resuscitation Research (e.g. Operation Brain Trauma Therapy), Dr. Patrick Kochanek, who initially set up the MRI collaboration with our group and served as an advisor for the entire project. Likewise, I presented a poster on my updated MRI and histopathology data from the project at this meeting.

- **How were the results disseminated to communities of interest?**

At the seven conferences listed above, I presented the data from this study in poster format at single day sessions that were open to all attendees, including representatives from the scientific industry sectors (e.g. lab supply companies). We also provided an illustrated abstract on our cytokine assay findings for the blasted rat brains to the USAMRMC for their FY17 Report to the Executive Agent (EA) on the Science and Technology Efforts and Programs Relating to the Prevention, Mitigation, and Treatment of Blast Injuries. This will be disseminated to the military research community in a newsletter format.

- **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

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

Other labs in the field of blast induced neurotrauma have shown interest in some of the novel methodologies behind our work, i.e., macrophage cell tracking by ¹⁹F-MRI; and thus, want to incorporate this techniques into their studies with advisement from us and the University of Pittsburgh's Animal Imaging Center.

- **What was the impact on other disciplines?**

While we are primarily providing our preliminary findings to only other labs in the field of blast induced neurotrauma. Other disciplines within science (e.g. nutritional research) have shown great interest in our work. This is mainly a result of our final findings that dietary omega-3 fatty acids have a modest ability at best to heal neuronal injuries resulting from blast induced neurotrauma. In the past, it has been frequently thought that dietary omega-3 fatty acids have potent anti-inflammatory and neuronal cell membrane building properties, which can overcome retina and brain injuries independent of the insult type and tissue extent. This study has clearly shown that omega-3s, like any drug, have limited physiological targets and therapeutic windows. For example, we found that it best targets microglia activation and expedites recovery only at the chronic stage. This is highly important for physicians who are considering to use omega-3s to treat severe neuronal injuries. We believe that they should considered providing a patient omega-3s as a long-term supportive therapy, in conjunction with standard neuroprotective drugs, and not as a first line intervention.

- **What was the impact on technology transfer?**

There is nothing to report, since this project does not involve development of a new technology.

- **What was the impact on society beyond science and technology?**

We are not officially releasing our final findings to the general public. As we are a DoD affiliated research laboratory, it is not our duty to provide the civilian population with recommendations on the use of omega-3 fatty acid supplements to treat blast induced neurotrauma to the visual system in human subjects. Also, our study was strictly a preclinical trial, as done in a lower-anatomy animal model (rats) and thus, is difficult to directly translate to the human condition. Our results in rats showing that omega-3s have a modest efficacy against blast injuries, must be verified in advanced studies, i.e., large animal (e.g. pigs) and human clinical trials at the University level. Only then can they be used by the medical profession to warn patients not to rely on increased intake of omega-3 fatty acids as a sole treatment approach. Dietary omega-3s have been a steadily growing nutritional fad for the prevention of many neurological afflictions, but with a rather generalized scientific basis. Instead, we believe omega-3s should be considered only as an adjunct supportive therapy for blast wave insults to the retina and brain.

- **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

- **Changes in approach and reasons for change**

There were no changes in experimental approach as found in the approved animal protocol during this entire study; however, we did deviate from the original grant proposal as follows:

1) Number of animals brought in for each experiment was increased to 12 - 18 rats, in order to compensate for the high number of animal deaths (i.e., up to 30%) incurred during the blast

exposures. These losses for the most part are unavoidable, since necropsy has shown that poly-trauma complications, i.e., brain, heart, lung, and/or liver damage, to be the primary cause of sudden death in the blasted rats. We have found that the only way to diminish this is to make sure the animal is fully secure in the holder during blasting to prevent additional losses from grave injuries due to movement and striking of objects as the blast wave passes by the test subject.

2) We had originally purposed doing pattern (p) and visually evoked brain potential (VEBP) ERGs for determining retinal ganglion cell / optic nerve and brain visual center deficits in the blasted animals. Due to procedure and equipment problems, we only analyzed the basic retinal signaling function of the rats, using full field flash ERG, for the two dietary groups at 3, 7, and 28 days out. The VEBP-ERG was found impractical, due to the special surgical efforts required to implant recording screws in the animal's skull over the occipital cortex. We tried some practice VEBP recordings, with limited success, on rats under a different project. It appears that positioning of the screws and lack of contact with muscle tissue is extremely important to obtain a good brain signal after light stimulus of the eyes. Additionally, we ran into constant problems with health impacts on the rats, i.e., gapping head wounds and infections. WRAIR Veterinary Medicine put a long-term halt on the VEBP-ERG experiments because of these animal welfare issues. While the p-ERG recordings are relatively simple to do, our current system also lacked the pattern stimulator video screens (i.e., an alternating checker board) necessary to carry out this test for retinal ganglion cell response. We incorrectly thought the instrument's current flash lamp already had this capability. The manufacturer (Diagnosys, LLC) eventually determined that the hard and software upgrades needed to do the p-ERGs were not possible with our older model instrument.

3) Cytokine levels in the plasma, brain, and retina of blasted rats were measured by multiplex immunoassay arrays (Luminex; R&D Systems, Inc). These arrays can determine the simultaneous concentrations of up to 17 rat specific factors, are rapid to complete (< 9 hours), run 96 samples at once, and are ultrasensitive down to the pico-molar range. We selected 10 of the available factors to assay, i.e., CXCL2, CXCL3, ICAM-1, IL-1- α , IL-4, IL-6, IL-18, TIMP-1, TNF- α , and VEGF. Originally, we had planned to analyze the samples using immunoassay micro-blot arrays (R&D Systems, Inc), which simultaneously detect 29 distinct types of cytokines / chemokines; however, while these arrays are lower in cost, they provide only a qualitative measurement of any changes.

4) In the first two years of the study, we had over focused on doing blasted animals under both dietary treatments groups for ERG, visual acuity, and cytokine immunoassay array analysis at 3 and 7 days out. Work up of blasted animals for MRI of the eyes (retina) and brain and subsequent histopathology had taken a back seat to this, due to our collaborator for the MRI analysis, Dr. T. Kevin Hitchens, moving his lab from Carnegie Mellon University to the University of Pittsburgh. He became the director of their Animal Imaging Center, which was actually an added advantage for supporting this project. His new lab was fully opened in June 2016. Thus, over the last year and a half of the study, we heavily focused on doing MRI and histopathology analysis of the rats at 3, 7, and 28 days out. The other outcome measures, i.e., visual acuity, ERG, and cytokine assays, were, however, completed for all of these end points.

5) We were not able fully complete histopathology analysis of eyes (retinas) and brains from rats taken at 3, 7, and 28 days post-blast, as originally purposed. Only a modest amount of samples at 3 and 7 days out were fully assessed (n = 5 - 12) for activated microglia and reactive astrocyte cell counts, i.e., Iba-1 and GFAP immunohistochemistry (IHC), respectively. This was due to a very slow return of eye and brains from the rats sent to the University of Pittsburgh for MRI analysis, since they became back logged on scanning the animals until the very end of the study. Indeed, they never returned to us any samples from the animals at 28 days post-blast that we had sent to them. Also, due to the delays, we never quantified the degree of neurodegeneration (e.g. axonal fiber tract degeneration) present in the retina and brain, via silver and H&E staining. Eventually, after their return, we will pursue histopathology analysis of the unfinished samples under other funding sources, in order to have complete data for publication purposes.

6) We had also injected some of the animals with fluorescein-labeled MRI contrast agent at 24 hours prior to all of the end points, to demonstrate via histopathology that it was localizing to macrophages

accumulating at eye (retina) and brain injury sites. Again, due to the prolonged delays in getting samples returned to us by the University of Pittsburgh following the MRI scanning, this assessment was never fully done. As above, however, we plan on eventually completing this task using other funding sources to have the data for inclusion in publications.

7) While we had purposed it as part of the histopathology work up, our attempts at eye (retina) and brain Glut-5 (glucose transporter 5) IHC for activated microglia showed a high amount of non-specific antibody binding to neurons in the rat brain sections (data not shown). Ultimately, we decided to drop this method as redundant with the Iba-1 IHC (activated macrophages and microglia) and MRI (macrophages alone) outcomes. Likewise, CD68 IHC on eye sections suggested that activated macrophages are completely absent in the blasted retinas at 3 days out (data not shown). We did see a modest buildup of macrophages in the brain optic tracts. Our MRI and Iba-1 IHC results strongly indicated that there is a massive infiltration of macrophages into the eyes for the two diet groups by 3 days post-blast. It is possible that the antibody used for the CD68 IHC is poorly reactive towards macrophages in the eye sections, due to altered epitope factors. It is known, however, that CD68 expression in macrophages can be highly tissue and/or injury specific, so this biomarker may not be useful. Thus, we decided to also drop full pursuit of CD68 IHC from the study.

8) For all statistical analyzes, we only did simple t-test comparisons between the shams and their corresponding blasted groups or just between the two dietary treatments. The comparisons were done always within the same end point post-blast and not across time. This was done for simplicity sake, as a preliminary screening to rapidly identify significant differences for the very large number outcome measures comprising the study. We realize, however, that the appropriate way to analyze the data is through ANOVA tests with repeated measures weighting, using comparisons across all treatment group and end point combinations. Thus, prior to future publication of the data from this study, we plan to hire an “in house” statistician to rigorously address this problem for us.

9) The animal protocol was amended three times with official review and approval of the changes by the WRAIR-IACUC and then the USAMRMC-ACURO. The first amendment was approved on 10 February 2016; and was a request to allow the surgical implantation of stainless steel skull screws in the blast exposed rats, to serve as attachment sites for recording electrodes used during the VEBP-ERG experiments (see above). We also added one new technician to the study, Andrew Bature, mainly to do blast exposure, visual acuity, ERG, and cytokine assay experiments on the animals. The second amendment was approved on 28 July 2017; and was a request to add 48 more animals to the protocol to help finish out the remaining MRI experiments. Throughout the study, we had a higher than anticipated mortality rate for the rats (~ 36%), during the double blast exposures; and thus, several months early, we used up the allotted number of animals on the protocol. This was mainly due to severe damage to their vital internal organs, e.g. lungs and heart, at the high blast over pressures that we were using along with a double hit exposure. The 48 extra animals allowed us to successfully finish the remaining experiments for the study with 16 rats to spare. The third amendment was approved on 27 September 2017, and was a request to add three new technicians to the study, David Bloodgood, Wafae Dirwech, and Jenifer Taskesen. Their responsibilities were mainly to assist as needed in carrying out the paraformaldehyde perfusions and tissue collections from the blast exposed rats.

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

1) Overall, the project fell outside of the main stream mission of the WRAIR Center for Military Psychiatry and Neuroscience Research, whose primary focus is on characterization and treatment of traumatic brain injuries alone. Thus, there is less drive for committing our institute's resources to studies involving peripheral neurosensory deficits (i.e., vision loss). There was also a concern that the project may compete with Army organizations having extensive ocular trauma task areas (e.g., USAISR, San Antonio, TX). Our center director when this study began, COL Maurice L. Sipos, was very adamant about me considering these points; however, we obtained a new center director, LTC

(P) Jeffery Thomas, who was more receptive to my eye research projects. His desire was that I continue my efforts with a sensible commitment and pursuit of additional funding. Likewise, we obtained a new institute commander, COL Deborah L. Whitmer, who in the past has engaged in eye research for the military, i.e., clinical diagnosis of laser injuries to the retina, and was very interested in supporting my research. Throughout the remainder of the study I made a strong effort to interact with LTC Thomas and COL Whitmer regarding the scope and future directions of my studies.

2) Due to problems with changing institutes and instrument failures / breakdowns, the University of Pittsburgh fell over one year behind schedule for doing MRI analysis of the eyes and brain for the placebo and fish oil treated rats at 3, 7, and 28 days post-blast. Thus, we requested and were granted a one year “no cost” extension from the USAMRMC for the project to catch up with this severe lag in effort. The last groups of MRI animals, however, were not finished scanning until almost the very end of the one year extension period and thereafter the data processing of the raw image files took several months to complete. This necessitated us to turn in the final grant report about a month and a half past the official due date of 29 December 2017. We had officially requested an extension of the report’s deadline, with the above justification; but never heard anything back from the USAMRMC, other than a warning from the program managers that the report was delinquent.

- **Changes that had a significant impact on expenditures**

1) We have greatly increased the group sizes of animals than originally purposed for each treatment and associated outcome measures. This was necessary to appropriately power the study for detecting statistically significant differences between groups. Additional animal and supply costs were covered by internal funds (USAMRMC-MOMRP) awarded to our lab chief Dr. Joseph B. Long.

2) We are using different immunoassay arrays than originally purposed to measure the cytokine / chemokine levels in plasma, brain, and retina from blast wave exposed rats. These new arrays are fully quantitative micro-titer plate style, as opposed to qualitative micro-blot, but are 3 times more expensive per kit (i.e., \$500 vs. \$1,500); however, in the long run they are more cost effective for the amount of samples that we have to do (i.e., 5 strip blots vs. a 96 well plate).

3) We submitted only a rather limited number of eye and brain samples for histopathology assessments (microscope slides) to the local company FD Neurotechnologies, due to this being an outcome measure done in conjunction (i.e., afterwards) with the MRI analysis of the rats (see sections above). Thus, these samples when returned to us in the future by the University of Pittsburgh, will be processed for histopathology under other grant funds held by our lab.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

- **Significant changes in use or care of human subjects**

The project does not involve the use of human subjects.

- **Significant changes in use or care of vertebrate animals.**

There is nothing to report; since through weekly monitoring of their body weights and blood glucose levels we found that there was no need for carrying out food restriction to control obesity or metabolic syndrome (pre-diabetes) in the rats, as we had originally thought would arise.

- **Significant changes in use of biohazards and/or select agents**

There is nothing to report, since this project does not involve the use of marked biohazards and/or select agents (e.g., infectious diseases and toxins / poisons). Syringe needles for animal injections and paraformaldehyde for tissue fixation are the only potential biohazards (low level) that are found in our experiments.

- **PRODUCTS:**

- **Publications, conference papers, and presentations**

1) Poster presentation entitled “Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves” was given at the National Capitol Area TBI symposium held at the NIH in Bethesda MD, on 4 - 5 April 2016. A copy of the abstract and poster is attached to the final report.

2) Poster presentation entitled “Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves” was given at the National Neurotrauma Society Symposium held in Lexington KY, on 25 - 30 June 2016. A copy of the abstract and poster is attached to the final report.

3) Poster presentation entitled “Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves” was given at the Military Health Systems Research Symposium (MHSRS) held in Kissimmee / Orlando FL, on 15 - 18 August 2016. A copy of the abstract and poster is attached to the final report.

4) Poster presentation entitled “Magnetic Resonance Imaging (^{19}F -MRI) based Tracking of Macrophage Infiltration in the Visual System of Rats following Exposure to Primary Blast Waves” was given at the National Capitol Area TBI symposium held at the NIH in Bethesda MD, on 08 - 09 March 2017. A copy of the abstract and poster is attached to the final report.

5) Electronic (E) poster presentation entitled “Characterization of Inflammation Induced by Exposure to Primary Blast Waves in Rats using ^{19}F MRI” was given at the International Society for Magnetic Resonance in Medicine (ISMRM) 27th Annual Meeting held in Honolulu HA, on 22 - 27 April 2017. A copy of the abstract and poster-slides is attached to the final report.

6) Poster presentation entitled “Magnetic Resonance Imaging (^{19}F -MRI) based Tracking of Macrophage Infiltration in the Visual System of Rats following Exposure to Primary Blast Waves” was given at the Arrow Head Traumatic Brain Injury 7th Annual Conference held in Washington DC (Arlington, VA), on 24 - 25 May 2017. A copy of the abstract and poster is attached to the final report.

7) Poster presentation entitled “Magnetic Resonance Imaging (^{19}F -MRI) Based Tracking of Macrophage Infiltration in the Visual System of Rats following Exposure to Primary Blast Waves” was given at the Military Health Systems Research Symposium (MHSRS) held in Kissimmee / Orlando, FL, on 27 - 30 August 2017. A copy of the abstract and poster is attached to the final report.

- **Journal publications.**

There is nothing to report; however, we are planning to publish a paper with the University of Pittsburgh on the MRI method alone to be submitted to the Journal of Magnetic Resonance in Medicine; and a separate paper on the rest of the study likely to be submitted to the Journal of Neurotrauma. The goal is to have these paper accepted for publication and in press within 2018.

- **Books or other non-periodical, one-time publications.**

There is nothing to report.

- **Other publications, conference papers, and presentations.**

Illustrated abstract entitled “Cytokine Responses in a Rat Model of Blast-induced Mild Traumatic Brain Injury (mTBI)” to be released in a newsletter by the USAMRMC as part of a FY17 Report to the Executive Agent (EA) on the Science and Technology Efforts and Programs Relating to the Prevention, Mitigation, and Treatment of Blast Injuries. A copy of the abstract is attached to the final report.

- **Website(s) or other Internet site(s)**

There is nothing to report.

- **Technologies or techniques**

There is nothing to report, since this project does not entail technology or technique development.

- **Inventions, patent applications, and/or licenses**

There is nothing to report, since this project does not entail invention development.

- **Other Products**

There is nothing to report.

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	James C. DeMar, Ph.D.
Project Role:	Principle Investigator - WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	34
Contribution to Project:	Directed and helped technicians execute all reported experiments.
Funding Support:	Geneva Foundation contractor – WRAIR

Name:	Joseph B. Long, Ph.D.
Project Role:	Co-Investigator – WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	6
Contribution to Project:	Management of personnel and budget for project. Consultant for results.
Funding Support:	Civilian government employee – WRAIR

Name:	T. Kevin Hitchens, Ph.D.
Project Role:	Co-Investigator - University of Pittsburgh, McGowan Institute
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	8
Contribution to Project:	Directed MRI analysis of rats; data interpretation
Funding Support:	Faculty - University of Pittsburgh

Name:	Lesley M. Foley
Project Role:	Technician - University of Pittsburgh, McGowan Institute
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	10
Contribution to Project:	MRI analysis of rats; data preparation and presentation
Funding Support:	Faculty - University of Pittsburgh

Name:	John G. Rosenberger
Project Role:	Technician - WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	30
Contribution to Project:	Feeding and gavaging (fish oil), body weight and blood glucose measurements, fresh tissue collections, MRI contrast agent infusions of rats. Data entry / analysis.
Funding Support:	Clinical Research Management contractor – WRAIR

Name:	Andrew B. Batuure
Project Role:	Technician - WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	30
Contribution to Project:	Visual acuity testing, ERG exams, cytokine immunoassay arrays, fresh tissue collections, and MRI contrast agent infusions of rats. Data entry / analysis.
Funding Support:	Oak Ridge Institute Research Education (ORISE) contractor – WRAIR

Name:	Donna M. Wilder
Project Role:	Technician - WRAIR
Researcher Identifier (e.g. ORCID ID):	Unknown
Nearest person month worked:	12
Contribution to Project:	Rat blast exposures, animal purchasing & care, and experiment scheduling.
Funding Support:	Clinical Research Management contractor – WRAIR

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

COL Thomas G. Oliver, M.D. (Co-PI; Walter Reed Army Hospital) and Patrick Kochanek, Ph.D. (consultant, Carnegie Mellon University) did not make significant work contributions to the project over the entire course of the study; and thus, are not cited above.

- **What other organizations were involved as partners?**

We have utilized the services of our outside collaborators at the University of Pittsburgh, McGowan Institute's Animal Imaging Center (Dr. T. Kevin Hitchens' lab) for the MRI analyses of our animals and the local company FD Neurotechnologies (Dr. Fu Du; Ellicott City, MD) for histopathology processing (microscope slides) of rat eyes (retinas) and brains. We had active CRADAs in place for the work done with both of these organizations.

- **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**

There is nothing additional to report, the efforts by our collaborators at the University of Pittsburgh (Dr. T. Kevin Hitchens' lab) have been extensively detailed in the sections above.

- **QUAD CHARTS:**

Quad Chart is attached.

- **APPENDICES:**

Attached are copies of abstracts and posters presented for the project at six local / national conferences, as well as an illustrated abstract for the USAMRMC FY17 Report to the Executive Agent (EA) on the Science and Technology Efforts and Programs Relating to the Prevention, Mitigation, and Treatment of Blast Injuries.

Elucidation of Inflammation Processes Exacerbating Neuronal Cell Damage to the Retina and Brain Visual Centers, as a Quest for Therapeutic Drug Targets, in a Rat Model of Blast Over Pressure Wave Exposure. Focus area: Mitigation and treatment of traumatic injuries, war-related injuries, and diseases to ocular structures and the visual system. Funding Opportunity Number: W81XWH-13-CRMRP-VRP-HDA



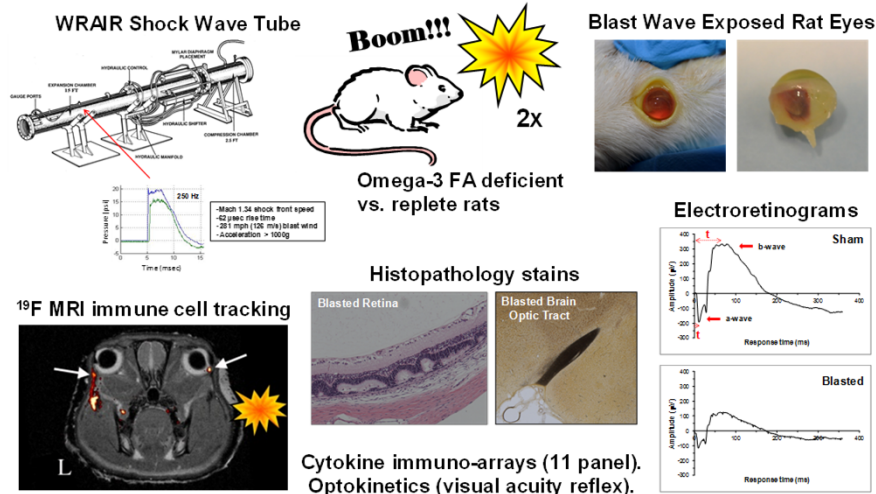
PI: James C. DeMar, Jr., Ph.D. Org: The Geneva Foundation Award Amount: \$249,998.00

Study/Product Aim(s)

- Blast overpressure (BOP) is a leading cause of vision loss in US soldiers, due to closed eye injuries (43% incidence with 26% involving the retina).
- Very few animal studies have characterized BOP induced visual system injuries or looked for drug therapeutics.
- We hypothesize that immune cell mediated processes play a central role in promoting neuronal cell death in the blasted retina and brain.
- Our objective is to monitor nature and timing of inflammatory processes in retina and brain visual centers of BOP exposed rats to discern drug targets and therapeutic windows. Dietary omega-3 fatty acid impact will be studied.

Approach

(1) Rats on an omega-3 fatty acid deficient or enriched diet exposed twice to blast waves (20 psi) using a shock tube. (2) At 3, 7, 14, and 28 days post-blast, inject with ^{19}F MRI tracer to in vivo label macrophages, and then perfusion fix. (3) Send to Univ. Pittsburgh for anatomical and cell tracking MRI of eyes and brain. (4) Histopathology of fixed eyes and brains for morphology, axonal degeneration, and activated immune cells. (5) Cytokine immunoassay arrays on fresh plasma, retinas, and brains. (6) Electroretinogram (retinal signaling) and optokinetic (visual acuity) tests done on all rats.



Accomplishments: Study was wrapped up by fully completing most of the proposed outcome measures at 3, 7, and 28 days post-blast. Final grant report was assembled.

Timeline and Cost

Activities	CY	14	15	16	17
Write animal protocol and submit to WRAIR-IACUC and USAMRMC-ACURO for approval.					
Complete 3 and 7 day post-blast time points in rats for all outcome measures.					
Complete 14 and 28 day post-blast time points in rats for all outcome measures.					
Wrap up data and then write final reports and publications.					
Estimated Budget (\$K)		\$125K	\$125K	\$000	\$000

Updated: 16 February, 2018 1 year, no cost extension approved; ends 29 Sept., 2017

Goals/Milestones

CY14 Goals – Initiate study; animal testing:

- Obtain a WRAIR-IACUC and USAMRMC-ACURO approved animal use protocol. Purchase rats, diets, and immunoassay array kits. Train on instrumentation.
- Blast rats, which are kept on omega-3 fatty acid deficient or enriched diets, and at 3 and 7 days post-injury do all outcome measures. (n = 4 - 22, each).
- Non-blasted rats will also be analyzed in same manner at 28 days out.

CY15 Goals – Complete study; drug targets and windows identified:

- Blast rats, kept on diets, and at 14 and 28 days post-injury do all outcome measures as above (n = 4 - 22, each). One year no cost extension needed?

Comments/Challenges/Issues/Concerns

- MRI of rats was achieved at 3, 7, and 28 days post-blast with solid group sizes.
- We will publish a methods paper for the MRI analysis in the Spring of 2018.
- The only outcome that fell short regarding completion is histopathology of eyes and brains. We will finish these samples using funding from other sources.

Budget Expenditure to Date

Actual Expenditure: \$226,747.83

Characterization of Inflammatory Processes in the Visual Systems of Rats Induced by Exposure to Primary Blast Waves

James DeMar, PhD¹, John Rosenberger, BS¹, Andrew Batuure, BS¹, Donna Wilder, BS¹, Meghan Mccuiston, BS¹, Patrick Kochanek, MD², Lesley Foley, BS³, and Kevin Hitchens, PhD³, and Joseph Long, PhD¹.

¹Walter Reed Army Institute of Research, Center for Military Psychiatry and Neuroscience Research, Silver Spring, MD.

²University of Pittsburgh School of Medicine, Safar Center for Resuscitation Research, Pittsburgh, PA.

³University of Pittsburgh, Animal Imaging Center, Pittsburgh, PA.

BACKGROUND: Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual centers from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, i.e., a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. **METHODS:** Adult male rats were maintained for one month on omega-3 fatty acid-enriched (via fish oil supplementation) versus -deficient diets. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to two closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). At 3, 7, 14, and 28 days post-blast the migration of macrophages to retina and brain injury sites was longitudinally tracked by magnetic resonance imaging (MRI; 19F-perfluorocarbon contrast agent) and neuronal cell dysfunction and neuroinflammation were confirmed through combined electroretinography (ERG), visual acuity (optokinetics), histopathology (Iba-1 and GFAP immunohistochemistry), and cytokine level (multiplex immunoassay arrays) outcome measures. **RESULTS:** Our findings reveal that retinal signaling impairments occur early post-blast injury (i.e., within 7 days), and are accompanied by neuronal cell degeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines. Dietary omega-3 fatty acids have thus so far shown slight, if any, ability to alleviate these acute injury events. **CONCLUSIONS:** In rats, blast wave exposure causes marked neuronal cell damage to the visual

system (retina and brain) that is associated with multi-faceted inflammatory processes. Despite having potent anti-inflammatory properties, omega-3 fatty acids did not readily alleviate these acute injury events. Chronic events, e.g. neuronal cell repair, may be more amenable to other functions of omega-3 fatty acids, such as membrane structural restoration. Thus, we plan to extend evaluations over more prolonged post-blast intervals. Overall, our mission is to provide data that will lead to discovery and animal testing of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army. SUPPORT: Funded by DoD grants from the MOMRP and USAMRMC / CDMRP, #: W81XWH-14-2-0178.

How to generate a reliable animal model of simulated blast wave induced neurotrauma to the visual system.

How to characterize inflammation processes and neuronal cell dysfunction / death in retinal and brain visual center injuries.

How to assess the efficacy of a nutritional based therapeutic, i.e., omega-3 polyunsaturated fatty acids, towards alleviating neuroinflammation and neurodegeneration in the retina and brain.

Characterization of Inflammatory Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves

¹James DeMar, ¹John Rosenberger, ¹Andrew Batuure, ¹William Rittase, ¹Donna Wilder, ¹Meghan Mccuiston, ²Patrick Kochanek, ³Lesley Foley, ³Kevin Hitchens, and ¹Joseph Long.

¹Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience Research, Walter Reed Army Institute of Research, Silver Spring, MD; ²Safar Center for Resuscitation Research and ³Animal Imaging Center, University of Pittsburgh, Pittsburgh, PA.



ABSTRACT

BACKGROUND: Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual centers from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory (e.g. a diet enriched in omega-3 polyunsaturated fatty acids) on blast injury vulnerability. **METHODS:** Adult male rats were maintained for one month on omega-3 fatty acid-enriched (via fish oil supplementation) versus -deficient diets. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to two closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). At 3, 7, 14, and 28 days post-blast the migration of macrophages to retina and brain injury sites was longitudinally tracked by magnetic resonance imaging (MRI; ¹⁹F-perfluorocarbon contrast agent) and neuronal cell dysfunction and neuroinflammation were confirmed through combined electroretinography (ERG), visual acuity (optokinetics), histopathology (Iba-1 and GFAP immunohistochemistry), and cytokine level (multiplex immunoassay arrays) outcome measures. **RESULTS:** Our findings reveal that retinal signaling impairments occur early post-blast injury (i.e., within 7 days), and are accompanied by neuronal cell degeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines. Dietary omega-3 fatty acids have thus far shown little, if any, ability to alleviate these acute injury events. **CONCLUSIONS:** In rats, blast wave exposure causes marked neuronal cell damage to the visual system (retina and brain) that is associated with multi-faceted inflammatory processes. Despite having potent anti-inflammatory properties, omega-3 fatty acids did not readily alleviate these acute injury events. Chronic events, e.g. neuronal cell repair, may be more amenable to other functions of omega-3 fatty acids, such as membrane structural restoration. Thus, we plan to extend evaluations over more prolonged post-blast intervals. Overall, our mission is to provide data that will lead to discovery and animal testing of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: DoD grants from the MOMRP and USAMRMC/CDMRP, #: W81XWH-14-2-0178.

BACKGROUND

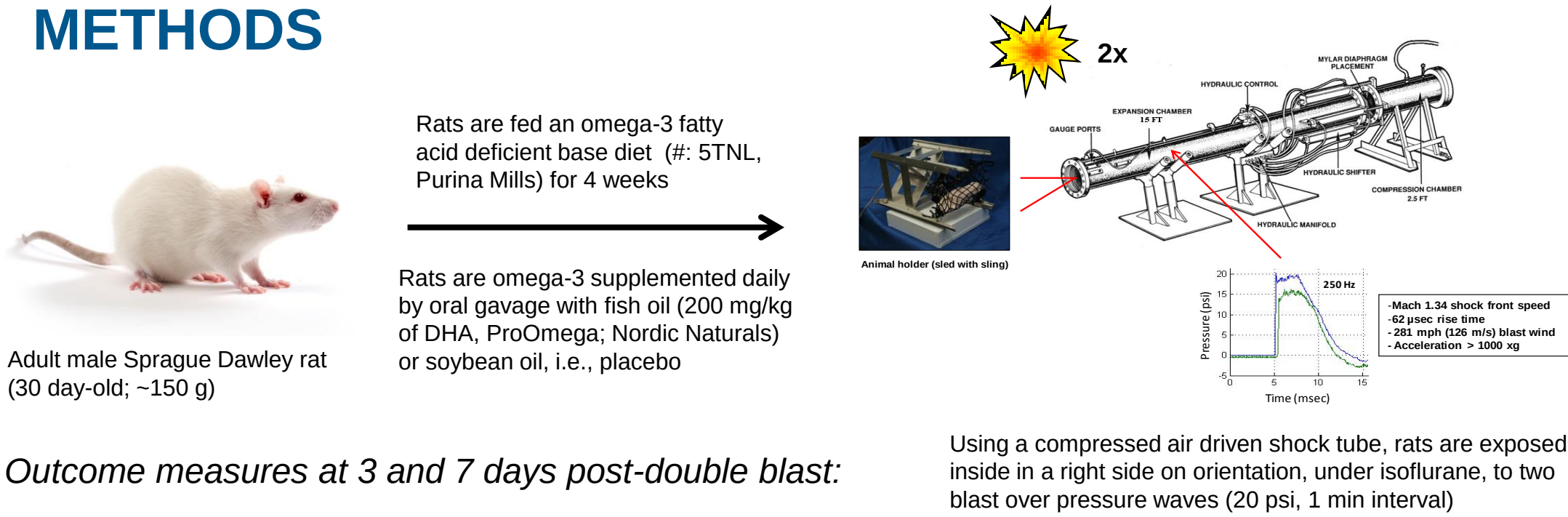
Blast exposure has caused since 2008 about 280,000 cases of traumatic brain injury in U.S. Soldiers, often with symptoms of vision loss (Capo-Aponte, 2012; Lemke, 2013). Of these patients, 43% display closed-eye injuries with 26% having retina damage (Cockerham, 2011). While soldiers wear protective goggles, they still can suffer eye injuries, e.g. blast wave penetration (Weichel, 2008). Few animal studies have tried to characterize visual system injuries as generated by high fidelity blast waves and/or have evaluated drug treatments (review by DeMar, 2016). Following blast wave injury, the brain and retina can undergo acute inflammation accompanied by immune cell activation, cytokine release, and neuronal cell degeneration (Serhan, 2010). Dietary omega-3 polyunsaturated fatty acids are converted to molecules, e.g. neuroprotectins and resolvins, which can suppress immune cell activation; and thus, may represent a practical therapeutic intervention (Serhan, 2008, 2011, 2012).

References: Capo-Aponte, 2012, Mil. Med. 177: 804-813; Cockerham, 2011, N. Engl. J. Med. 364: 2172-2173; DeMar, 2016, Front. Neurol. 7: 59 (1-12); Lemke, 2013, JAMA Ophthalmol. 131: 1602-1609; and Serhan, 2008, 2010, 2011, & 2012, British J. Pharm. 153: 5200-5215, Am. J. Pathol. 177: 1576-1591, Curr. Top. Med. Chem. 11: 629-647, & Chem. Rev. 111(10) 5922-5943; and Weichel, 2008, Curr. Opin. Ophthalmol. 19: 519-525.

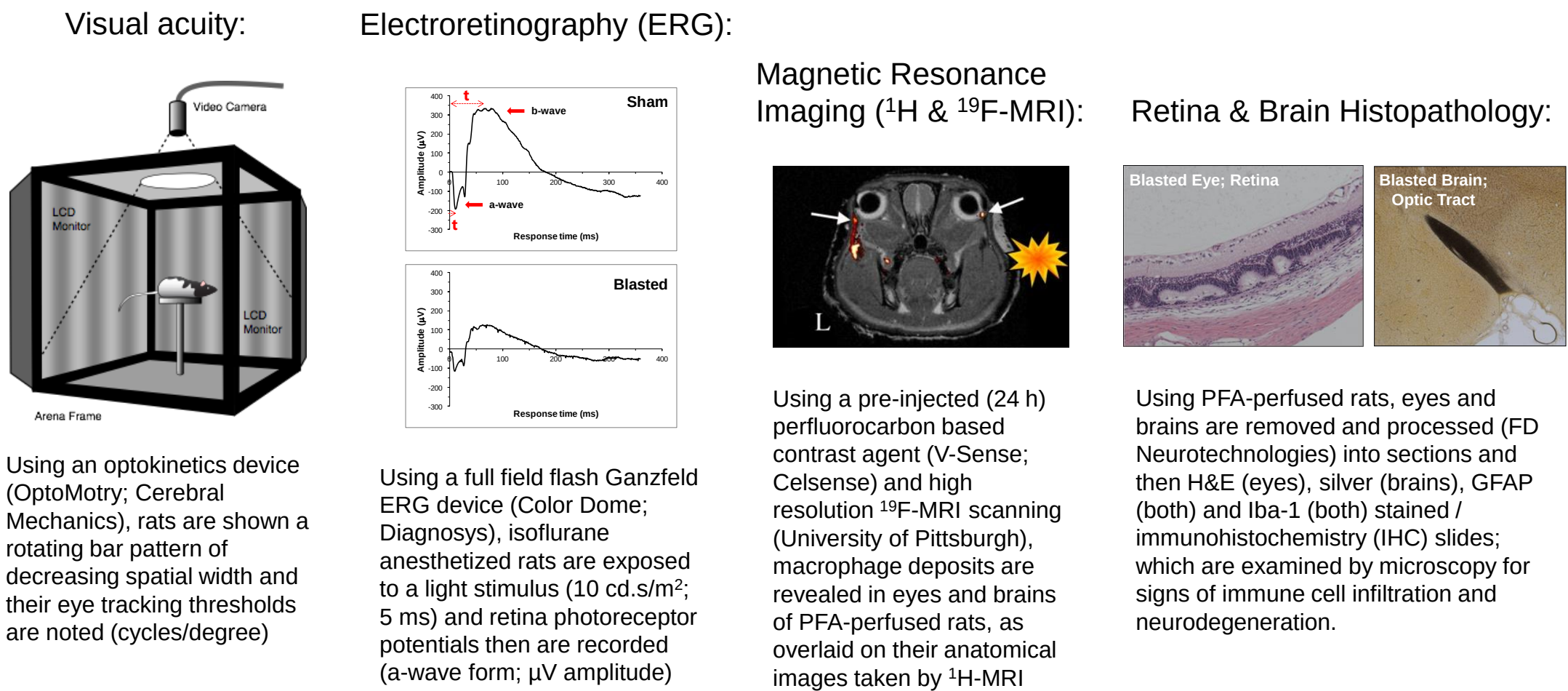
In our study, rats were maintained on omega-3 fatty acid deficient or enriched diets and then exposed to high fidelity simulated blast waves in a shock tube; and the resulting visual dysfunction and underlying neuroinflammation and neurodegeneration in the brain and retina was characterized by:

- 1) Visual acuity (optokinetics) and electroretinography (ERG).
- 2) Cytokine levels and Magnetic Resonance Imaging (MRI) based macrophage tracking.
- 3) Histopathology (H&E and silver stains; and GFAP & Iba-1 IHC).

METHODS



Outcome measures at 3 and 7 days post-double blast:



Brain cytokines:

Using terminal exsanguination, under isoflurane anesthesia, rats were sacked and whole brains collected and frozen. Thawed tissue was dissected into mid-brain containing the superior colliculus, i.e., a visual processing center, and the cerebellum as a known blast-injury site control. Cytokine levels of brain regions were determined, using protein lysates and immunoassay arrays (rat specific 10-plex; Luminex; R&D Systems). Values for cytokines were expressed as concentrations (pg/mg), after normalization against total brain protein (BCA assay; Thermo-Scientific).

DATA and RESULTS

Double blast impact on visual acuities and electroretinography (ERG) at 3 and 7 days post-exposure; both treatment groups show similar deficits in spatial resolution and retinal signaling function:

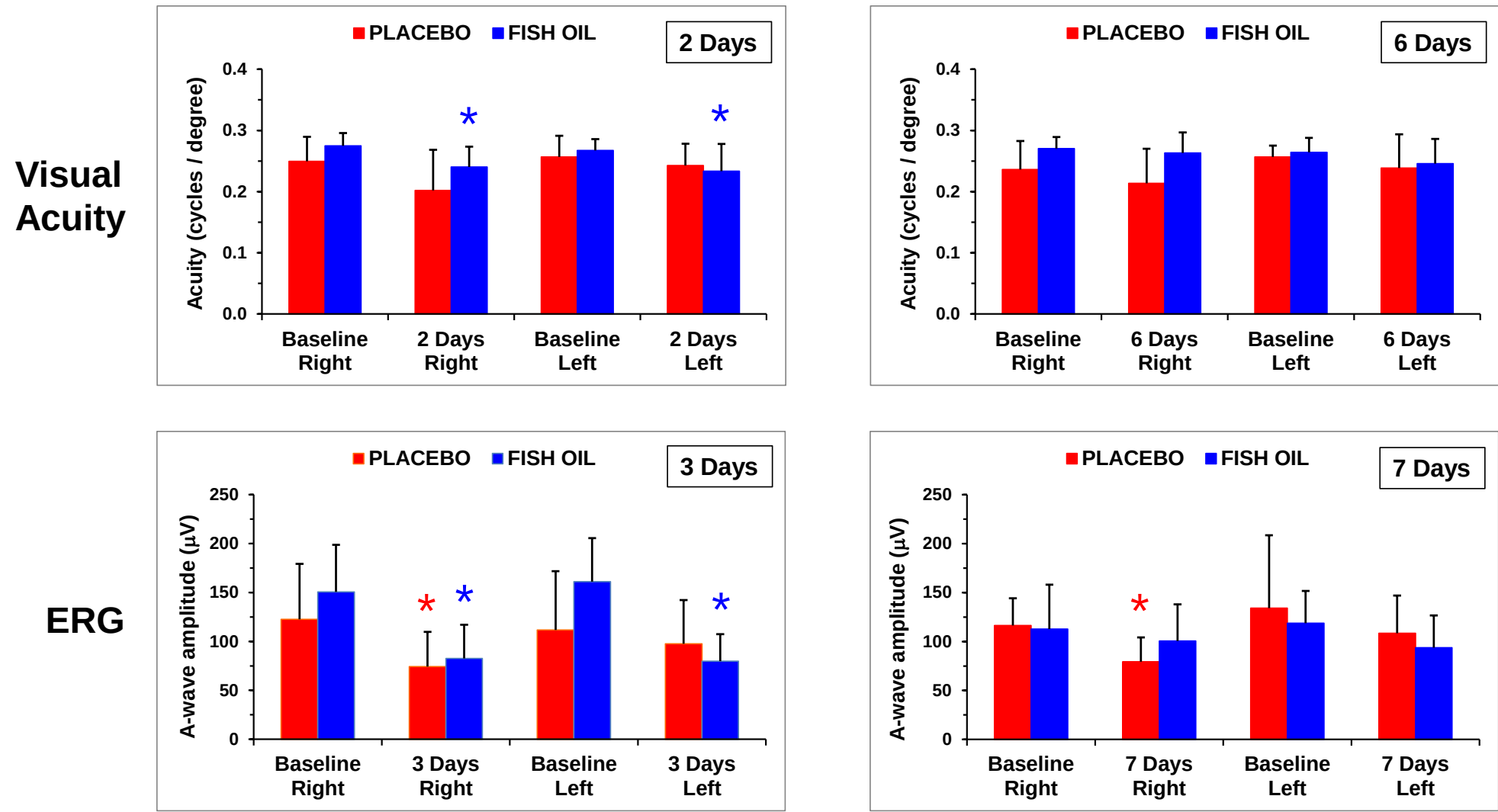


Figure 1. Visual acuities (cycles/degree) and ERG amplitudes (μ V; a-wave) of rats (left and right eyes) fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets at baseline and 2 - 3 and 6 - 7 days post-blast. Group sizes: n = 11 and 12 and n = 7 and 11, respectively. * p $\leq$ 0.05; significant difference from baseline, as by t-test.

Double blast impact on brain cytokines at 3 days post-exposure; both treatment groups show a similar increase in regional brain levels of specific cytokines:

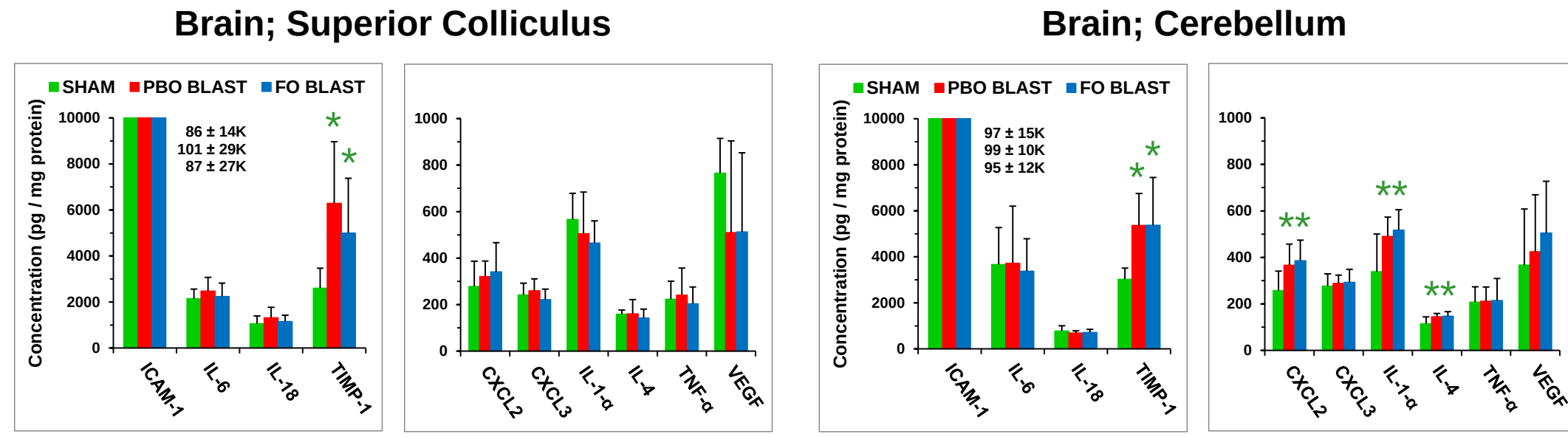


Figure 2. Brain cytokine levels (pg/mg) of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets, at 3 days post-blast. Shams were non-injured and not given omega-3 fatty acids. Group sizes: n = 7, 8, and, 7 each, respectively, for superior colliculus and cerebellum regions. *p $\leq$ 0.05; significant difference from shams (non-blasted), as by t-test. PBO = Placebo and FO = Fish Oil treatments. 10 cytokines were assayed.

Double blast impact on eye (in situ) ¹⁹F-MRI based macrophage tracking at 3 days post-exposure; both treatment groups show a similar presence of immune cell infiltration in the injured eyes:

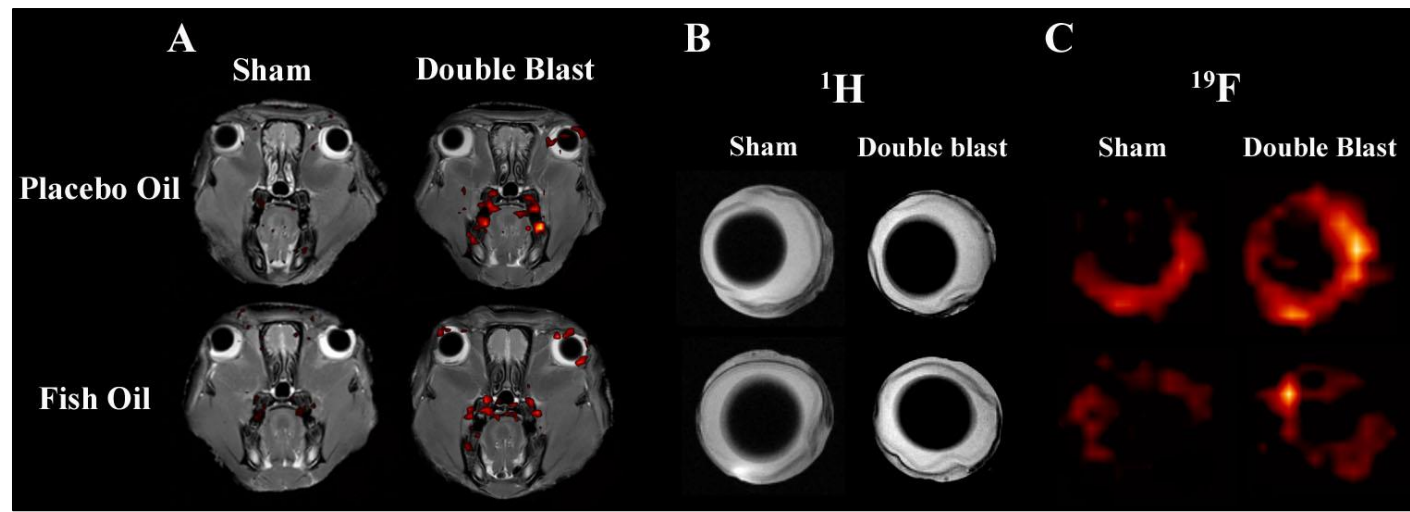


Figure 3. ¹H-MRI anatomical and ¹⁹F-MRI macrophage tracking analysis of rats fed omega-3 deficient (placebo) and enriched (fish oil) diets, for shams or at 3 days post-blast. In situ images of head (A) and merged eyes (B, C) of four representative animals. ¹⁹F signal is shown in red pseudo color (A, C).

Double blast impact on eye (retina) and brain (optic tract) histopathology at 7 days post-exposure; both treatment groups show a similar degree of neurodegeneration and immune cell activation:

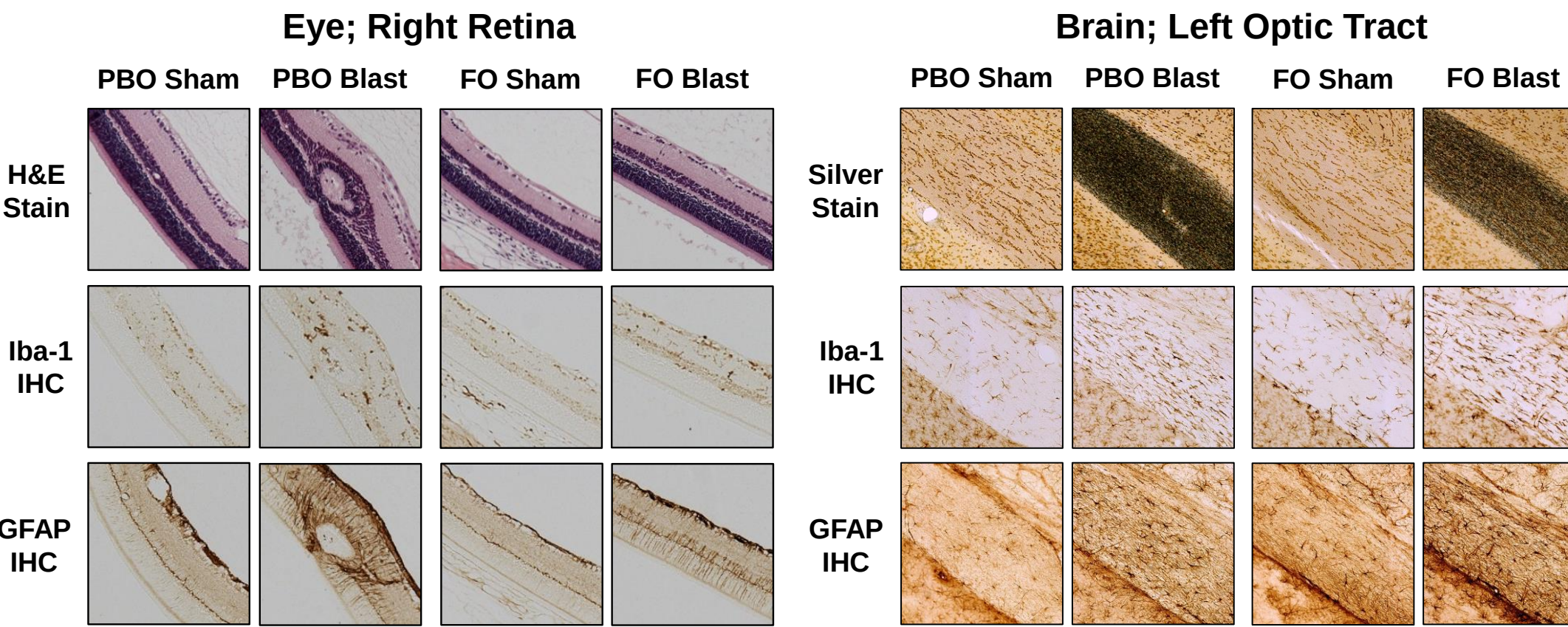


Figure 4. Histopathology of eyes (right retina) and corresponding brains (left optic tract) from four representative rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets, for shams or at 7 days post-blast. PBO = Placebo and FO = Fish Oil treatments. Magnification is 10 - 20x.

CONCLUSIONS

Our current findings with visual acuity and electroretinography (ERG) testing in rats reveal that retinal signaling impairments occur early post-blast injury (i.e., within 7 days). These deficits are accompanied by neurodegeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines, as obtained by immunoassay arrays, magnetic resonance imaging (MRI), and histopathology. Dietary omega-3 fatty acids, as given by high dose fish oil, have so far shown little, if any, ability to alleviate these injury events, despite having potent anti-inflammatory properties. Reason for lack of a robust treatment effect is unclear, but may be reflective of the injury time frame examined i.e., acute phase. Thus, we plan to extend evaluations over more prolonged post-blast intervals.

Magnetic Resonance Imaging (¹⁹F-MRI) Based Tracking of Macrophage Infiltration in the Visual System of Rats following Exposure to Primary Blast Waves

James DeMar, PhD¹, John Rosenberger, BS¹, Andrew Batuuere, BS¹, Donna Wilder, BS¹, Meghan Mccuiston, BS¹, Franco Rossetti, PhD¹, Patrick Kochanek, MD², Lesley Foley, BS³, and Kevin Hitchens, PhD³, and Joseph Long, PhD¹.

¹Walter Reed Army Institute of Research, Silver Spring, MD.

²University of Pittsburgh School of Medicine, Pittsburgh, PA.

³University of Pittsburgh, Pittsburgh, PA.

BACKGROUND: Blast injury has emerged as arguably the greatest threat to Warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual centers from explosion shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a rodent model of high fidelity simulated blast. Our hypothesis is that immune cells mediate the extent of blast-induced neuronal cell death in retina and brain visual centers. Our objective is to longitudinally monitor the nature and timing of immune cell infiltration in the retina and brain after blast, so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, i.e., a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. **METHODS:** Adult male rats were maintained for one month on omega-3 fatty acid-enriched (fish oil supplemented) versus -deficient diets. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to two closely-coupled repeated blast over pressure waves (20 psi, 8 msec duration, 1 min interval). At an early stage post-blast (3 and 7 days), rats (n = 8) were euthanized and perfused with saline and paraformaldehyde. Migration of macrophages to retina and brain injury sites was tracked in situ by magnetic resonance imaging (MRI) of the carcasses, using a ¹⁹F-labeled perfluorocarbon contrast agent that was selectively taken up by macrophages following intravenous administration at 24 hours prior to end points. Images for macrophage deposits (¹⁹F-MRI) were overlaid with those of the structural anatomy (¹H-MRI). Eye and brain sections were histopathologically evaluated to detect the presence of activated immune cells and degenerating neurons. **RESULTS:** MRI of blasted rats revealed, regardless of diets, that extensive macrophage infiltration occurred in both eyes along with some brain accretion (e.g. mid-brain). Immunohistochemistry (CD68, Iba-1, and GFAP) confirmed the eyes (retinas) and brain (optic tracts) contain abundant activated macrophages, microglia, and astrocytes. Cell morphology stains (H&E and silver) also showed ongoing neurodegeneration to be present in corresponding regions. **CONCLUSIONS:** In rats, MRI and histopathology showed blast wave exposure causes early onset neuronal cell damage to the visual system that was associated with increased immune cell activities. Despite having potent anti-inflammatory properties, omega-3 fatty acids did not diminish these acute injury events. Chronically activated immune cells may represent a softer target. Thus, we plan to extend evaluations over more prolonged post-blast intervals. Overall, our mission is to provide data to assist in discovery of new drug treatments for blast-induced neurotrauma sustained to US Army personnel. **SUPPORT:** Grants awards from MOMRP and USAMRMC/CDMRP, #: W81XWH-14-2-017.

KEY OBJECTIVES

How to utilize a reliable rodent model of simulated blast wave induced neurotrauma to the visual system.

How to characterize immune cell activation and neurodegeneration in retinal and brain injuries.

How to assess the efficacy of essential nutrients, i.e., dietary omega-3 fatty acids, as a neuroprotectant.

Magnetic Resonance Imaging (^{19}F -MRI) based Tracking of Macrophage Infiltration in the Visual System of Rats following Exposure to Primary Blast Waves



ABSTRACT

BACKGROUND: Blast injury has emerged as arguably the greatest threat to Warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual centers from explosion shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a rodent model of high fidelity simulated blast. Our hypothesis is that immune cells mediate the extent of blast-induced neuronal cell death in retina and brain visual centers. Our objective is to longitudinally monitor the nature and timing of immune cell infiltration in the retina and brain after blast, so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, i.e., a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability.

METHODS: Adult male rats were maintained for one month on omega-3 fatty acid-enriched (fish oil supplemented) versus -deficient diets. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to two closely-coupled repeated blast over pressure waves (20 psi, 8 msec duration, 1 min interval). At an early stage post-blast (3 and 7 days), rats (n = 8) were euthanized and perfused with saline and paraformaldehyde. Migration of macrophages to retina and brain injury sites was tracked in situ by magnetic resonance imaging (MRI) of the carcasses, using a ^{19}F -labeled perfluorocarbon contrast agent that was actively taken up by macrophages following intravenous administration at 24 hours prior to end points. Images for macrophage deposits (^{19}F -MRI) were overlaid with those of the structural anatomy (^1H -MRI). Eye and brain sections were histopathologically evaluated to detect the presence of activated immune cells and degenerating neurons.

RESULTS: MRI of blasted rats revealed, regardless of diets, that extensive macrophage infiltration occurred in both eyes along with some brain accretion (e.g. mid-brain). Immunohistochemistry (CD68, Iba-1, and GFAP) confirmed the eyes (retinas) and brain (optic tracts) contain abundant activated macrophages, microglia, and astrocytes. Cell morphology stains (H&E and silver) also showed ongoing neurodegeneration to be present in corresponding regions.

CONCLUSIONS: In rats, MRI and histopathology showed blast wave exposure causes early onset neuronal cell damage to the visual system that was associated with increased immune cell activities. Despite having potent anti-inflammatory properties, omega-3 fatty acids did not diminish these acute injury events. Chronically activated immune cells may represent a softer target. Thus, we plan to extend evaluations over more prolonged post-blast intervals. Overall, our mission is to provide data to assist in discovery of new drug treatments for blast-induced neurotrauma sustained to US Army personnel.

SUPPORT: Grants awards from MOMRP and USAMRMC/CDMRP, #: W81XWH-14-2-017.

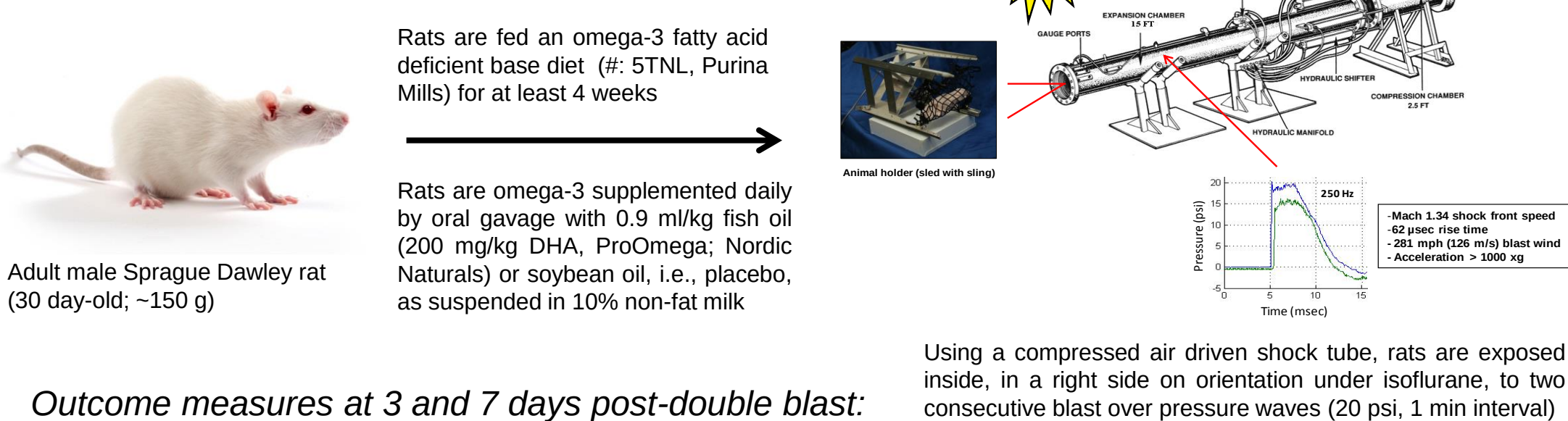
BACKGROUND

Blast exposure has caused since 2008 about 280,000 cases of traumatic brain injury in U.S. Soldiers, often with accompanying vision loss symptoms (Capo-Aponte, 2012; Lemke, 2013). Of these patients, 43% display closed-eye injuries with 26% having retina damage (Cockerham, 2011). While soldiers wear protective goggles, they still can suffer eye injuries, e.g. blast wave penetration (Weichel, 2008). Few animal studies have tried to characterize visual system injuries as generated by high fidelity blast waves and/or have evaluated drug treatments (review by DeMar, 2016). Following blast wave injury, the brain and retina can undergo acute inflammation accompanied by immune cell activation, cytokine release, and neuronal cell degeneration (Serhan, 2010). Dietary omega-3 polyunsaturated fatty acids are converted to molecules, e.g. neuroprotectins and resolvins, which can suppress immune cell activation and thus, may represent a practical therapeutic intervention (Serhan, 2008, 2011, 2012).

References: [Capó-Aponte](#), 2012, Mil. Med. 177: 804-813; [Cockerham](#), 2011, N. Engl. J. Med. 364: 2172-2173; [DeMar](#), 2016, Front. Neurol. 7: 59 (1-12); [Lemke](#), 2013, JAMA Ophthalmol. 131: 1602-1609; and [Serhan](#), 2010, 2010, 2011, & 2012, British J. Pharm. 153: 5200-5215, Am. J. Pathol. 177: 1576-1591, Curr. Top. Med. Chem. 11: 629-647, & Chem. Rev. 111(10) 5922-5943; and [Weichel](#), 2008, Curr. Opin. Ophthalmol. 19: 519-525.

In our study, rats were maintained on a long chain omega-3 fatty acid deficient or enriched (fish oil) diets and then exposed to high fidelity simulated blast waves in a shock tube. The resulting underlying neuroinflammation and neurodegeneration in the brain and retina was characterized at 3 days post-exposure by Magnetic Resonance Imaging based macrophage tracking (^{19}F -MRI) and at 3 and 7 days out by histopathology (H&E and silver stains; and CD68, GFAP, and Iba-1 immunohistochemistry).

METHODS



Outcome measures at 3 and 7 days post-double blast:

Magnetic Resonance Imaging (^1H & ^{19}F -MRI):

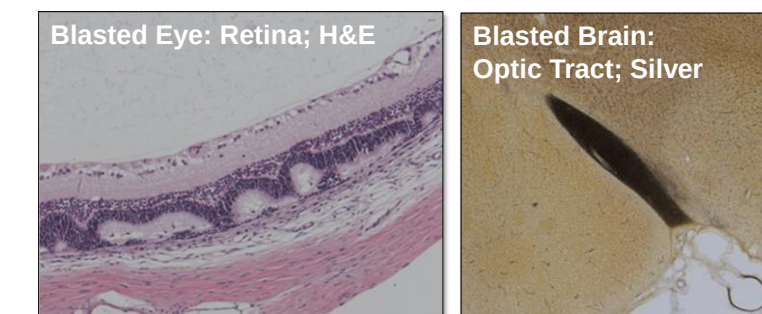


At 24 hours prior to the end point, rats are intravenously infused with a MRI contrast agent, which is a ^{19}F -labeled perfluorocarbon emulsion (VSense-1000; Celsense, Pittsburgh, PA) that is actively taken up by immune cells, mainly macrophages. This material provides a unique tracer for inflammation. Agent is given via lateral tail veins using a butterfly catheter and syringe pump at 3 ml/kg and 0.4 ml/min. Rats are euthanized at 3 days post-blatt by exsanguination and perfused with saline and PFA. Fixed carcasses are subjected by the University of Pittsburgh's Animal Imaging Center to high resolution ^{19}F -MRI scans of the eyes and brains. Macrophage deposits are revealed by overlaying the ^{19}F -signal as heat maps in "hot iron" pseudo-color, with anatomical images taken by ^1H -MRI.

University of Pittsburgh's MRI Machine Parameters:

- 1) Bruker, Biospec AVANCE III; 7.0, 9.4, and 11.7 Tesla (T) spectrometers.
- 2) 64 x 64 mm FOV, 2 mm slices, and resolution range of ~ 0.3 - 1 mm.
- 3) 25 slices and 24 ROI captured at 7.0T; 155 and 90 at 9.4T; and 4 and 1 at 11.7T.

Retina & Brain Histopathology



Eyes and brains from PFA-fixed rats at 3 and 7 days post-blast are prepared for histopathology assessments by FD Neurotechnologies (Ellicott City, MD). Eyes are horizontally sectioned (5 μ m) and subjected to hematoxylin & eosin (H&E) staining or GFAP and Iba-1 immunohistochemistry (IHC). Brains are coronal sectioned (30 μ m) for silver staining and CD68, GFAP, and Iba-1 IHC. Sections are examined by microscopy for neuroinflammation and neurodegeneration in the retina and brain optic tracts. H&E and silver reveal morphological perturbations and axonal fiber tract degeneration, respectively, characteristic of neuronal cell death. CD68 and Iba-1 are both cell markers of macrophages. Iba-1 also shows activated microglia by highlighting shape changes, i.e., non-ramified. GFAP expression and cellular processes are increased in activated astrocytes. Immune cells are quantified in captured images using Media Cybernetics Image-Pro Plus 7 software (Rockville, MD), as cell densities (counts per μ m²) averaged over 3 fields at 20x power.

DATA and RESULTS

Double blast impact on eye and brain macrophage accumulation at 3 days post-exposure; both dietary treatment groups show similar inflammation intensities on ¹⁹F-MRI and ¹H-MRI scans:

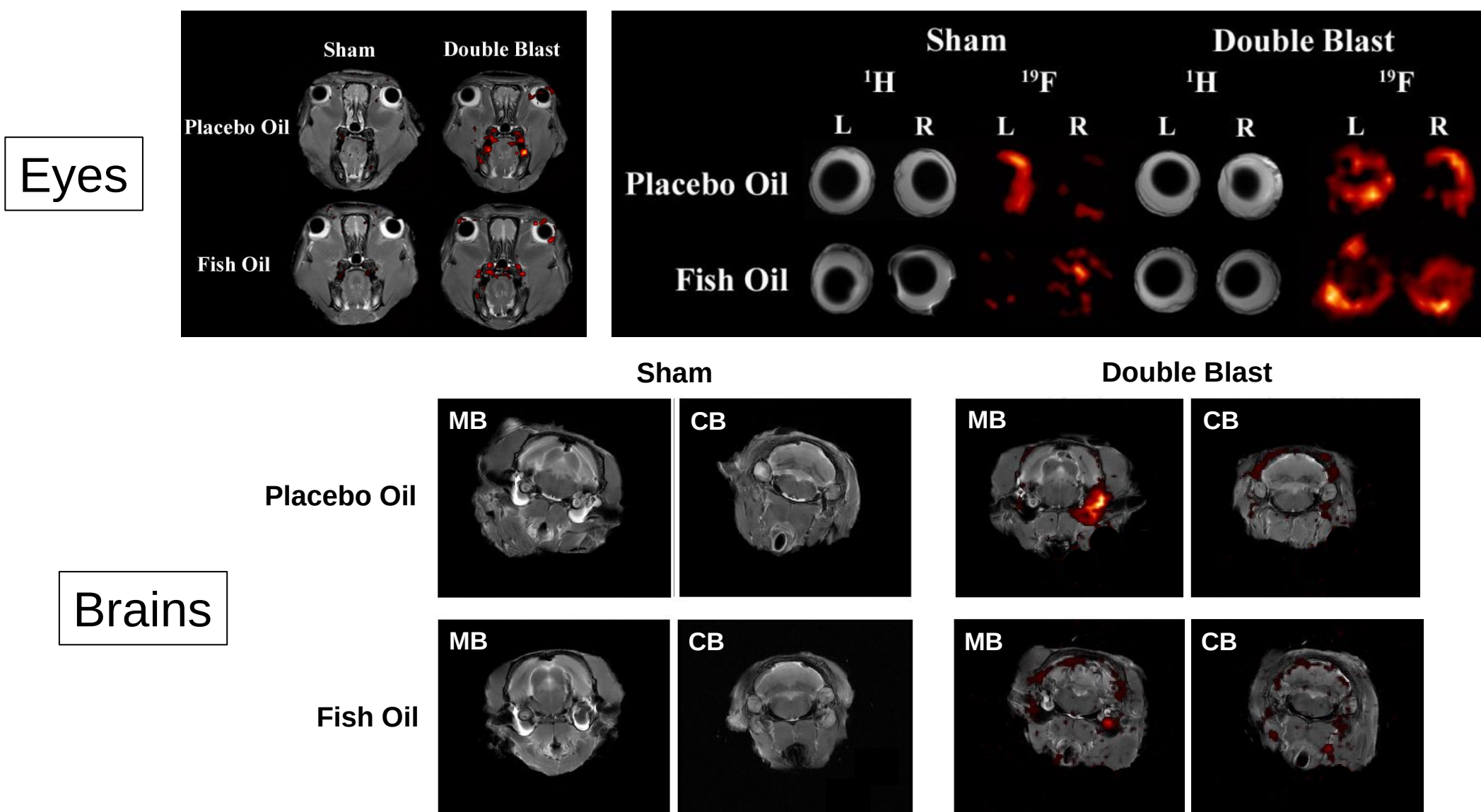


Figure 1. ^1H -MRI anatomical and ^{19}F -MRI macrophage tracking in rats. In situ images (7.0 & 11.7T) of eyes and brains (cerebellum; CB and mid-brain; MB regions) of representative sham and blasted animals (placebo and fish oil fed). ^{19}F -signal, as shown in "hot iron" pseudo-color, suggests marked immune cell infiltration is occurring in the blasted eyes and brains, despite dietary treatments. Brain signal is mainly near the meninges and skull bones.

Double blast impact on macrophage accumulation in eyes and related skull bones at 3 days post-exposure; both dietary treatment groups show a similar degree of inflammation:

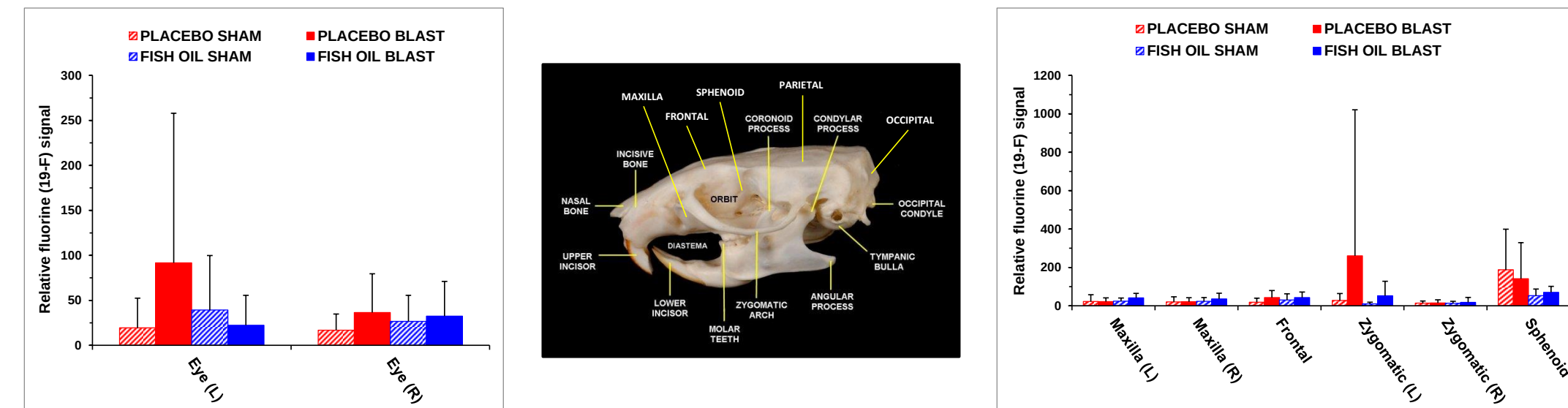


Figure 2. Bar graph for ^{19}F -MRI signals, as an indication of macrophage accumulation, found in placebo versus fish oil treated rats following double blast exposure or as shams (placebo = red and fish oil = blue, $n = 7, 10, 8$, and 12). Separate graphs are shown for eyes and the bones making up the surrounding orbital socket. Diagram is shown for bones of the rat skull. No significant differences were detected between the treatment groups.

Double blast impact on eyes at 3 days post-exposure; ¹⁹F-MRI macrophage cell tracking results agree with retina histopathology for neurodegeneration and immune cell infiltration:

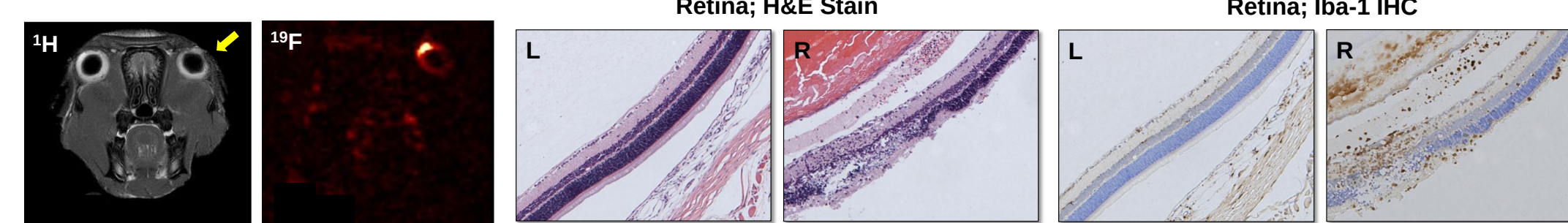


Figure 3. MRI and histopathology of a representative placebo oil treated rat at 3 days post-blast. In situ ^1H and ^{19}F -MRI (anatomical and macrophage images) of the rat's head is shown at a coronal plane passing through both eyes. Retina histopathology for the corresponding left and right eyes is shown for H&E stain for degenerating neurons (e.g. photoreceptors) and Iba-1 IHC for microglia and macrophages.

Double blast impact on eye (retina) and brain (optic tract) histopathology at 7 days post-exposure; both dietary treatment groups show a similar degree of neurodegeneration and immune cell activity:

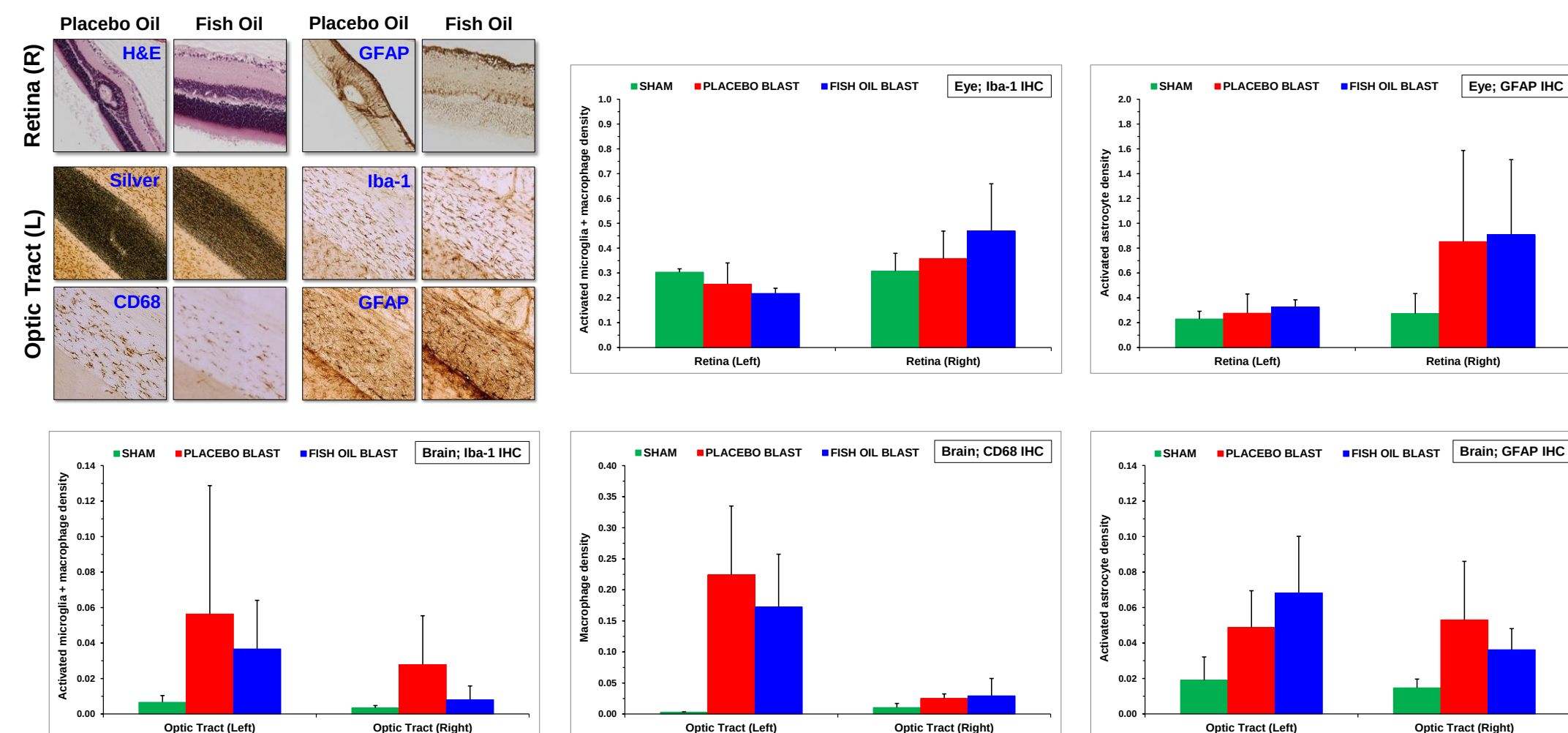


Figure 4. Histopathology of eyes (retina) and brains (optic tract) from representative placebo and fish oil treated rats at 7 days post-blast. Microscope images are shown for retinas and optic tracts under H&E and/or silver stain (degenerating neurons) and Iba-1, CD68, and GFAP IHC (activated microglia + macrophages, macrophages, and activated astrocytes, respectively). Retinal Iba-1 images are not shown here (see Fig. 4C), but treatments appeared similar. Bar graphs for immune cell densities (counts per μm^2) of retina and optic tracts (left and right) are also given (lumped shams = green, placebo = red, and fish oil = blue; $n = 2, 3, 3$, respectively. Current group sizes do not justify statistical comparisons.

CONCLUSIONS

Our current findings with ¹⁹F-MRI macrophage tracking and histopathology in rats reveal that immune cells involved in neurodegeneration and neuroinflammation processes, i.e., macrophages, microglia, and astrocytes, have accumulated in the retina and brain by 7 days following exposure to blast waves. Dietary omega-3 fatty acids, as given by high dose fish oil, have so far shown little, if any, ability to alleviate these injury events, despite having potent anti-inflammatory properties targeting immune cell activities. Reason for lack of a robust treatment effect is unclear, but may be reflective of the injury time frame examined i.e., acute phase. Thus, we plan to extend evaluations over more prolonged post-blast intervals.

Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves

¹James C. DeMar, ¹John G. Rosenberger, ¹Andrew B. Batuure, ¹Donna M. Wilder, ¹Meghan A. Mccuistion, ²Lesley M. Foley, ²Patrick M. Kochanek, and ²T. Kevin Hitchens, and ¹Joseph B. Long.

¹Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience Research, Walter Reed Army Institute of Research, Silver Spring, MD 20910; ²University of Pittsburgh, Pittsburgh, PA 15261.

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual center from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, i.e., a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Adult male rats were maintained for one month on omega-3 fatty acid-enriched (via fish oil supplementation) versus -deficient diets. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). At 3, 7, 14, and 28 days post-blast the migration of macrophages to retina and brain injury sites was longitudinally tracked by magnetic resonance imaging (MRI; 18F-contrast agent) and neuronal cell dysfunction and neuroinflammation were confirmed through combined electroretinography (ERG), visual acuity (optokinetics), histopathology (immunohistochemistry), and cytokine level (immunoassay arrays) outcome measures. Our findings reveal that retinal signaling impairments occur early post-blast injury (i.e., within 3 days), and are accompanied by neuronal cell degeneration in the retina and brain visual centers along with macrophage accretion, activated microglia and astrocytes, and increased cytokines. Dietary omega-3 fatty acids have thus so far shown little ability to alleviate these acute injury events. Overall, our mission is to provide data that will ultimately lead to discovery and animal testing of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: This work is supported by DoD grant awards from the MOMRP and USAMRMC / CDMRP (#: W81XWH-14-2-0178).

Outcome measures: None have studied the interplay of damage to the retina and brain. Thus, there is an urgent need to do advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role in dictating the extent of neuronal cell death in retina and brain following blast. Using adult rats exposed to shock tube generated blast waves, our objective is to longitudinally monitor up to 28 days the nature and timing of immune cell guided inflammatory processes in the injured retina and brain as to discern potential drug targets and therapeutic windows. We are also examining the impact of interventions known to be anti-inflammatory, i.e., giving the rats a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Overall, our mission is to provide data that will ultimately lead to discovery of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: DoD grant awards from the MOMRP and USAMRMC / CDMRP/ Vision Research Program #: W81XWH-14-2-0178.

Introduction

❑ Blast injuries have resulted since 2008 in more than 280,000 cases of traumatic brain injury in U.S. Soldiers, with frequent symptoms of vision loss (Capo-Aponte, 2012; Lemke, 2013).

❑ While brain visual centers can be affected, 43% display closed-eye injuries with 26% having retina damage (Cockerham, 2011).

❑ Soldiers are issued protective goggles but still suffer eye injuries, such as from blast wave penetration (Weichel, 2008).

❑ Few animal studies have tried to characterize visual system injuries as generated by rigorously simulated blast waves; and only three have evaluated drug treatments (review by DeMar, 2016).

❑ Following blast wave injury, the brain and retina can undergo acute inflammation accompanied by immune cell activation, cytokine release, and neuronal cell degeneration (Serhan, 2010).

❑ Metabolites of dietary omega-3 polyunsaturated fatty acids can suppress immune cell activation (Serhan, 2008, 2011, 2012).

References:

Capó-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
DeMar et al., 2016; Front. Neurol. Accepted pending revision.
Lemke et al., 2013; JAMA Ophthalmol. 131(12): 1602-1609.
Serhan et al., 2008, 2010, 2011, & 2012; British J. Pharm. 153: 5200-5215, Am. J. Pathol. 177(4) 1576-1591, Curr. Top. Med. Chem. 11(6) 629-647, & Chem. Rev. 111(10) 5922-5943.
Weichel et al., 2008; Curr. Opin. Ophthalmol. 19(6): 519-525.

Aim of Study

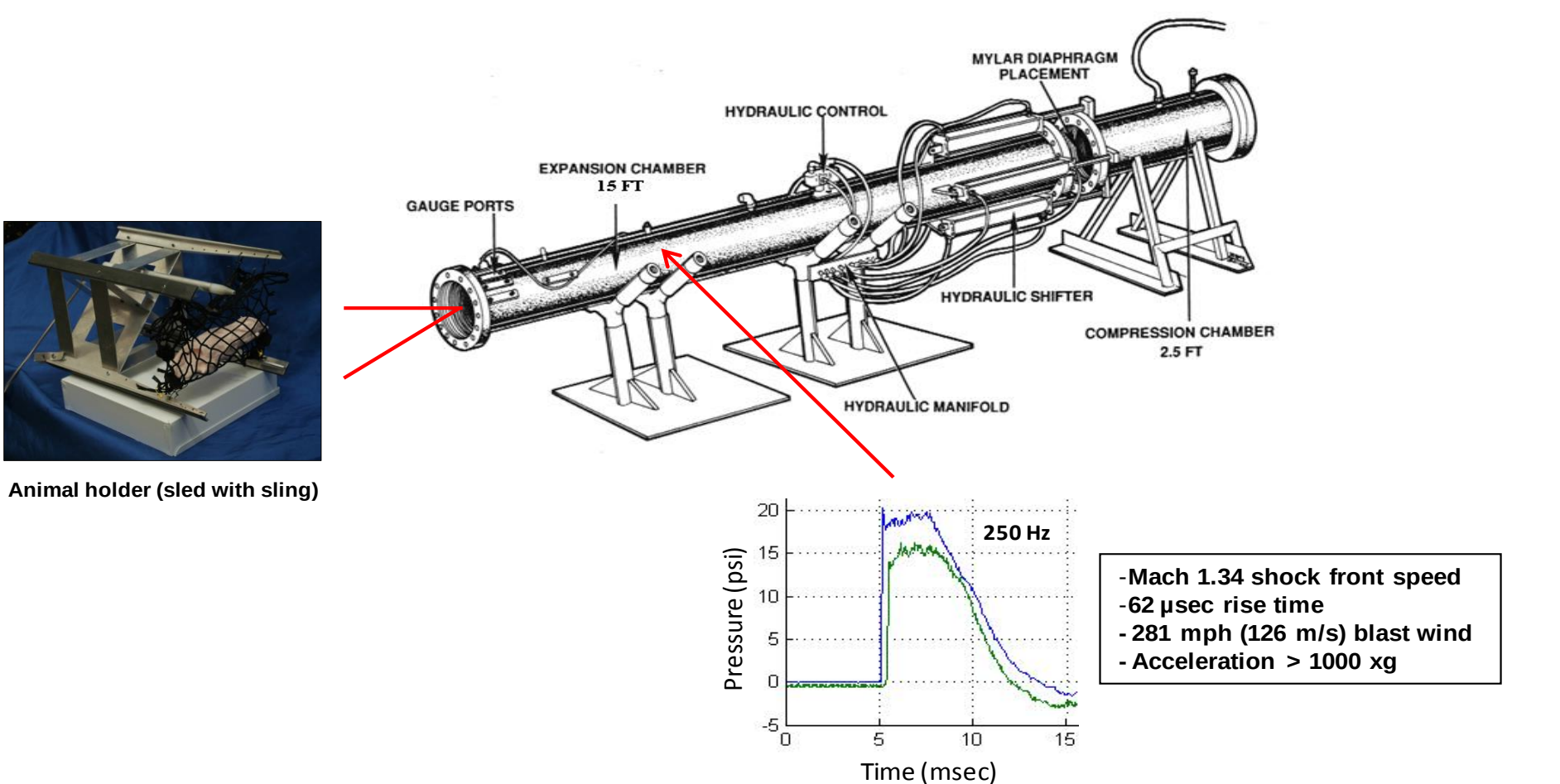
In rats raised on omega-3 fatty acid deficient or enriched diets and then exposed to high fidelity simulated blast waves, characterize up to 28 days out the visual dysfunction and underlying neuro-inflammation / degeneration due to retina and brain injures, as by:

- 1) Visual acuity (optokinetics) and electroretinography (ERG).
- 2) Bloodwork, tissue fatty acids and cytokines, and Magnetic Resonance Imaging (MRI) based immune cell tracking.
- 3) Histopathology (H&E and silver stains; Iba-1 and GFAP IHC).

provide 200 and 273 mg/kg of DHA and EPA. Placebo controls are given an equal volume of soybean oil.

Simulation of Primary Blast Wave Injuries:

❑ Rats are exposed under isoflurane anesthesia to two blast over pressure waves, i.e., double blast (20 psi; 1min interval), in a right-side on position, using a compressed air driven shock tube.



Visual Acuity (Optokinetics):

Using an optokinetics device (Optomotry; Cerebral-Mechanics), rats are put on a pedestal in a chamber, where a rotating bar pattern is shown on four LCD monitors. Rotation speed is increased stepwise to narrow the bar's width; and visual acuity threshold (cycles/degree) is found, when animal ceases head-eye tracking movements (nystagmus). Separate eye acuities are determined by driving the rotation in opposite directions.

Electroretinography (ERG):

Rats are dark adapted for 16 h. Using a full field flash Ganzfeld ERG device (Color Dome; Diagnosys), rats are placed under isoflurane, pupils are drug-dilated, and electrodes are put on eyes (recording), cheeks (reference), and tail (ground). Eyes are flashed with light (10 cd.s/m²; 5 msec); and evoked retina photoreceptor potentials are recorded (a-waveform).

Bloodwork, Tissue Fatty Acids and Cytokines, and ¹⁹F-MRI Based Immune Cell Tracking:

Rat blood (cardiac puncture) is submitted to WRAIR Clinical Pathology for Complete Blood Count (CBC) and chemistry panel. Total fatty acid profiles of fresh liver are obtained, using lipid extraction and GC/MS (7890A/5975C; Agilent) methods. Cytokine levels of fresh brain are found, using protein lysates and immunoassay arrays (Luminex; R&D Systems). Macrophage immune cell deposits are revealed in eyes and brains of PFA-fixed rats, using a pre-injected (24 h) ¹⁹F-contrast agent (V-Sense; Celsense) and high resolution MRI scanning (Univ. of Pittsburgh).

Histopathology (H&E, Silver, Iba-1, and GFAP):

Following transcardial perfusion of rats with PFA, fixed eyes and brains are processed (FD Neurotechnologies) into sections and then H&E (eyes) and silver, Iba-1, and GFAP (brains) stained / immunohistochemistry (IHC) slides; which are examined by microscopy for immune cell infiltration and neurodegeneration.

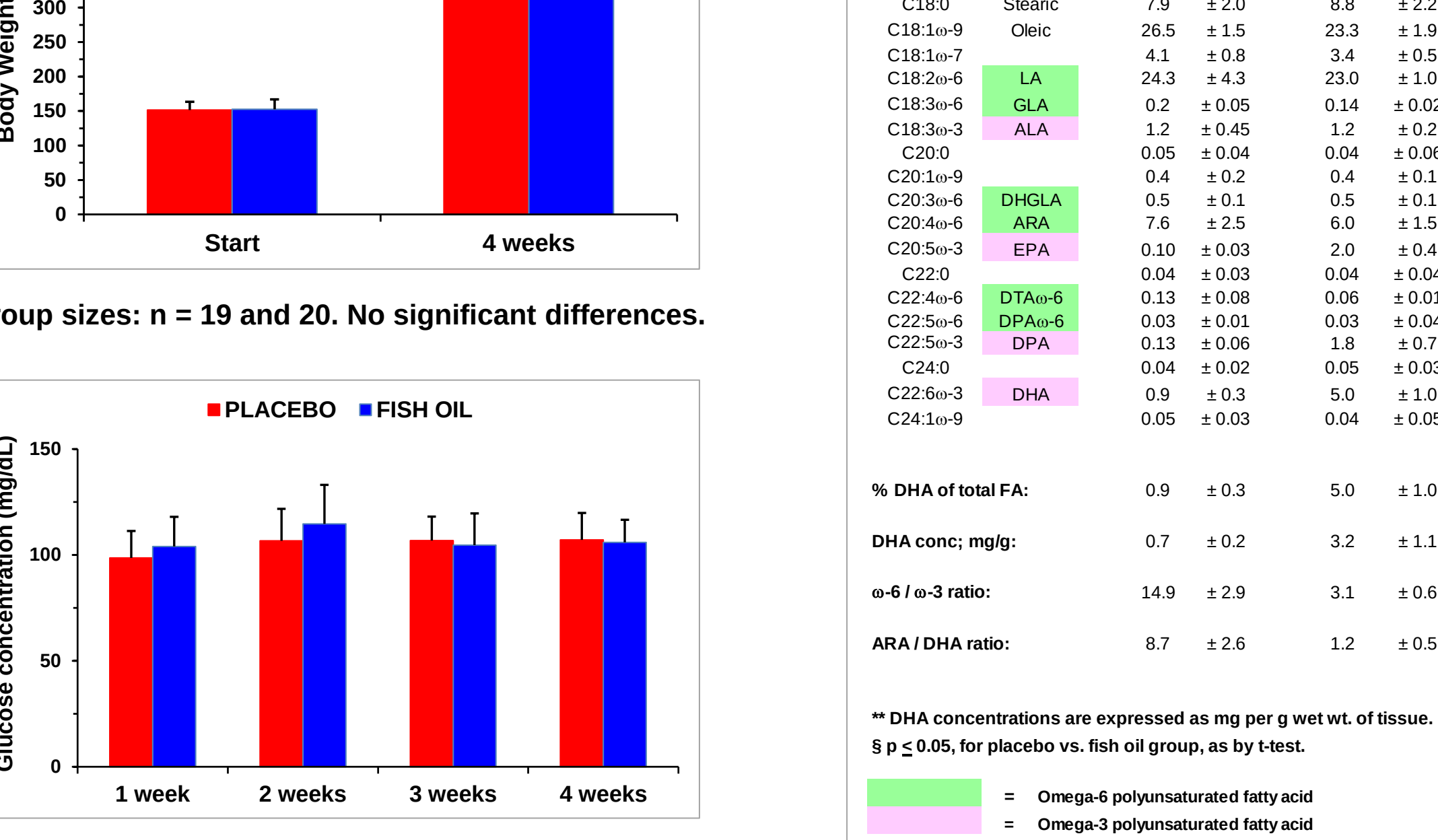


Figure 1. Body weights, blood glucose, and liver total fatty acids of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets for at least 4 weeks. Blood glucose was obtained by tail stick and a handheld diabetic monitor (Contour; Bayer); normal rats is 105 ± 13 mg/dL (n = 10).

Double Blast Impact on % Survival, Righting Reflex, and Terminal Bloodwork:

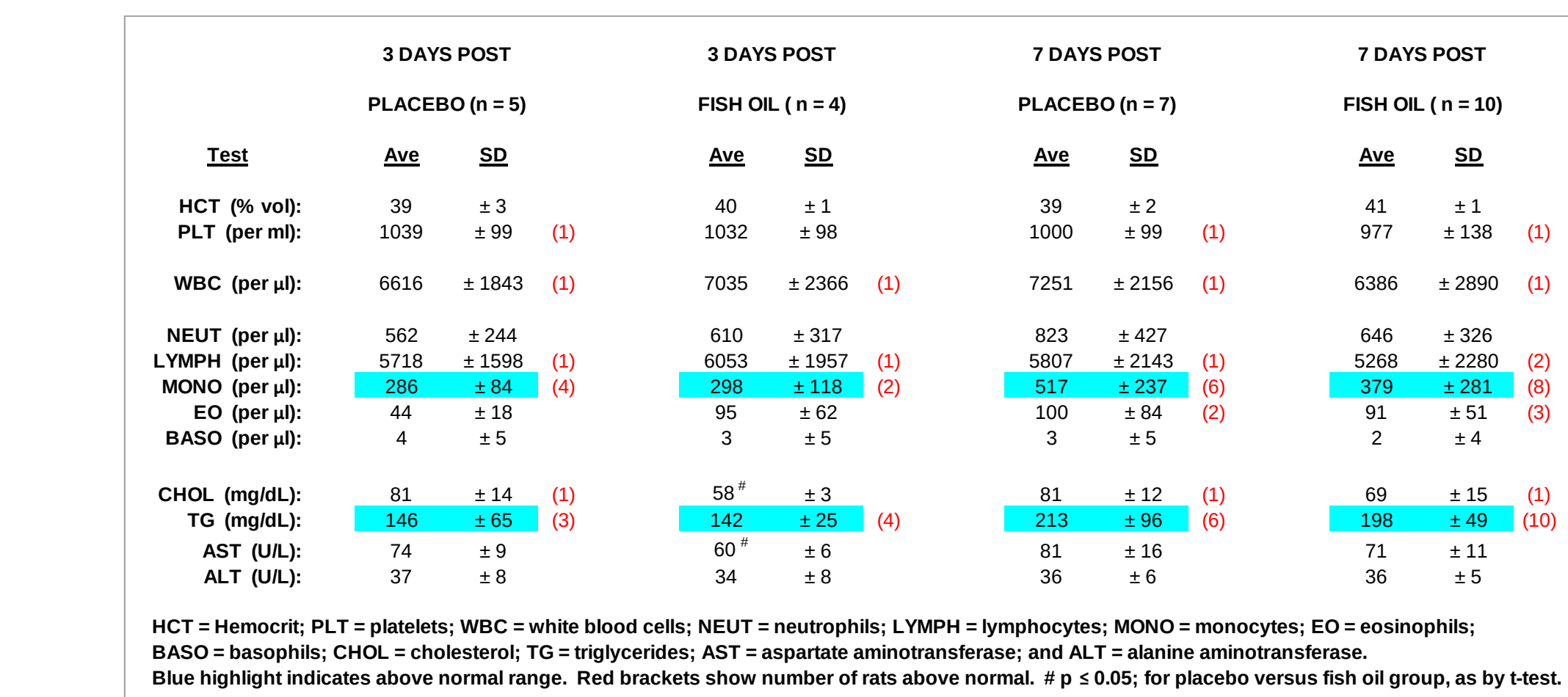
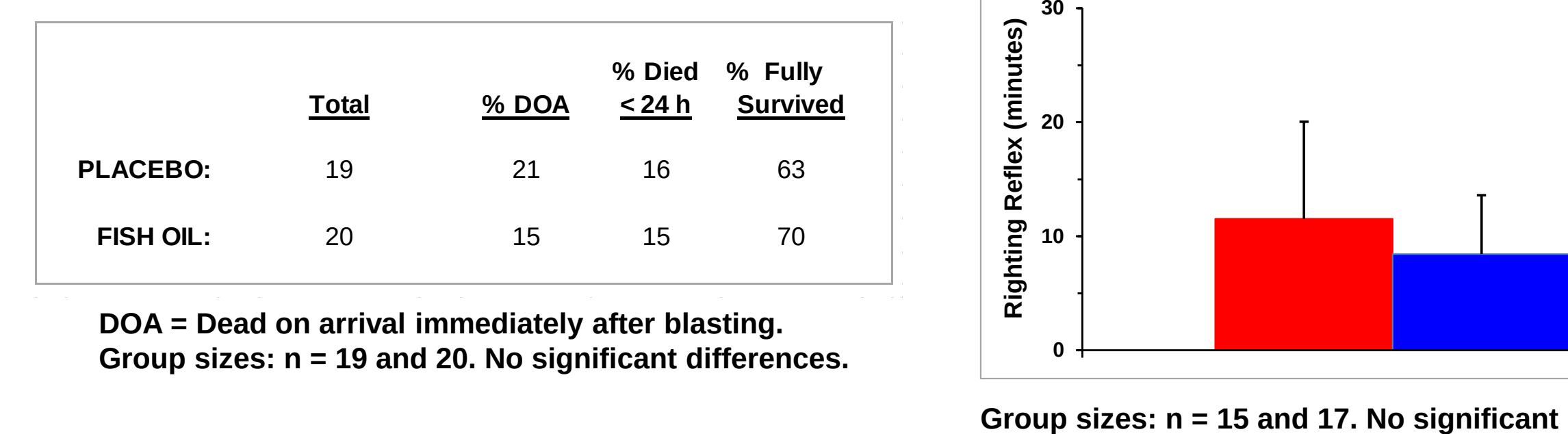


Figure 2. Percent survival, righting reflex, and bloodwork of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets at 3 and 7 days post-blast. WRAIR Clinical Pathology flagged any abnormal bloodwork.

Double Blast Impact on Visual Acuities:

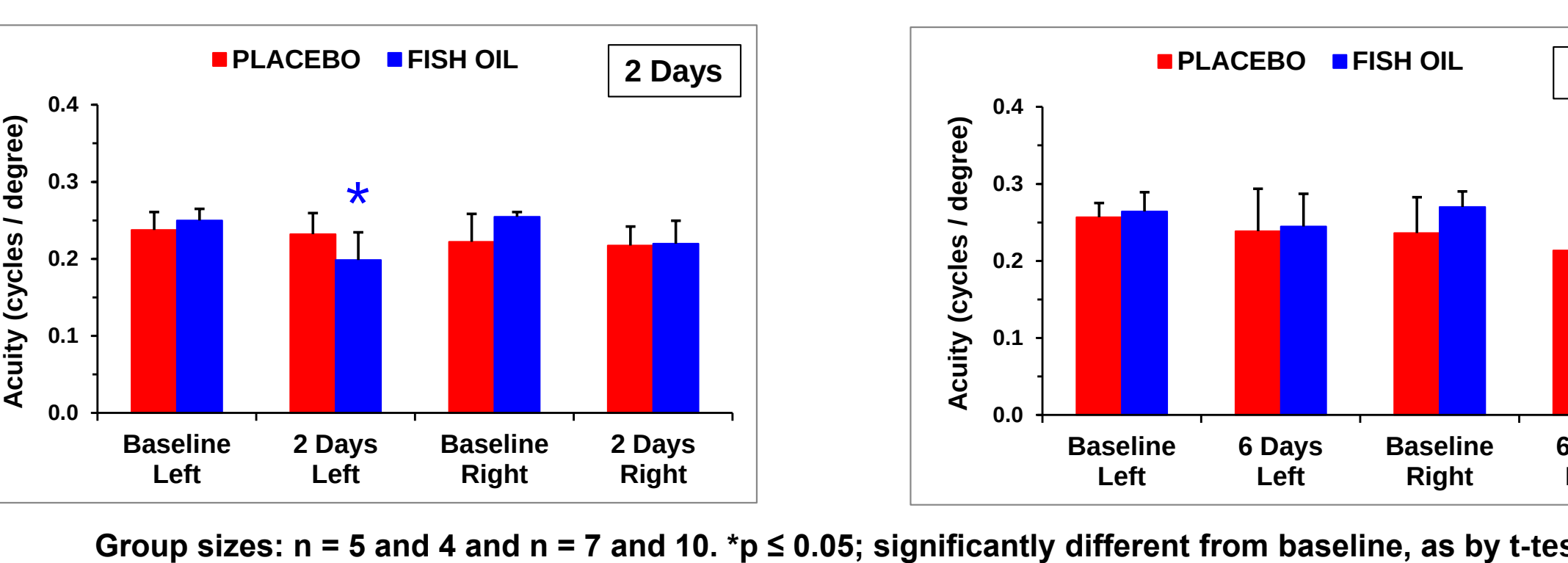


Figure 4. Visual acuity responses (cycles/degree) of rats (left and right eyes) fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets at baseline and 3 and 7 days post-blast.

Magnetic Resonance Imaging (¹⁹F-MRI) based Tracking of Macrophage Infiltration in the Visual System of Rats following Exposure to Primary Blast Waves

¹James C. DeMar, ¹John G. Rosenberger, ¹Andrew B. Batuure, ¹Donna M. Wilder, ¹Meghan A. Mccuiston, ²Lesley M. Foley, ²Patrick M. Kochanek, and ²T. Kevin Hitchens, and ¹Joseph B. Long.

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Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual center from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast injury. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast exposure. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, e.g. a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Adult male rats were raised for one month on an omega-3 fatty acid-deficient chow ("Typical American diet"; Test Diet, Purina Mills) versus one that was enriched by daily oral supplementation with ocean fish oil. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). Shams were also placed on the diets, but received only anesthesia exposure. At an acute stage post-blast, i.e., 3 days, the rats (n = 8 per treatment) were euthanized and paraformaldehyde perfused. Migration of macrophages to retina and brain injury sites was tracked in situ by magnetic resonance imaging (MRI) of the carcasses, using a ¹⁹F-labeled, i.e., perfluorocarbon, contrast agent (VS1000; Celsense) that was selectively taken up by macrophages following intravenous administration 24 hours before the end point. Animals were whole body imaged on a 9.4T spectrometer (AVIII HD, Bruker Biospin) and then the excised eyes and brains reimaged at 11.7T for higher resolution assessments. Images for macrophage deposits (¹⁹F-MRI) were overlaid with standard scans of structural anatomy (¹H-MRI) and signal density "heat" maps constructed. MRI analysis of blasted rats revealed that extensive macrophage infiltration occurred inside the globe of both eyes along with brain accretion mainly in the cerebellum, mid-brain, and surrounding meninges. Ocular macrophage accretion was more robust than that in the brain, consistent with the eyes being externalized; and thus, more susceptible to blast wave damage. Immunohistochemistry (CD68, Iba-1, and GFAP) confirmed that the eyes (retinas) and brain, especially the optic tracts, contain abundant activated macrophages, microglia, and astrocytes. Cell morphology stains (H&E and silver) also showed there to be ongoing neurodegeneration present in the corresponding retinal and brain regions.

Dietary omega-3 fatty acids, however, have shown so far a very modest ability, if any, to alleviate the majority of these acute neuropathological events. Thus, we plan to extend our study to chronic time points post-blast, i.e., up to 28 days post-injury, to look for a delayed therapeutic effect of omega-3 supplementation in promoting resiliency of neural tissues towards blast exposure. Overall, our mission is to provide data that will ultimately lead to discovery and animal testing of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: This work is supported by DoD grant awards from the MOMRP and USAMRMC / CDMRP (#: W81XWH-14-2-0178).



ABSTRACT

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual center from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast injury. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast exposure. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, e.g. a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Adult male rats were raised for one month on an omega-3 fatty acid-deficient chow (“Typical American diet”; Test Diet, Purina Mills) versus one that was enriched by daily oral supplementation with ocean fish oil. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). Shams were also placed on the diets, but received only anesthesia exposure. At an acute stage post-blast, i.e., 3 days, the rats (n = 8 per treatment) were euthanized by exsanguination and perfused with saline and paraformaldehyde. Migration of macrophages to retina and brain injury sites was tracked in situ by magnetic resonance imaging (MRI) of the carcasses, using a ¹⁹F-labeled, i.e., perfluorocarbon, contrast agent (VS1000; Celsense) that was selectively taken up by macrophages following intravenous administration 24 hours before the end point. Animals were whole body imaged on a 9.4T spectrometer (Biospec AVANCE III, Bruker) and then the excised eyes and brains reimaged at 11.7T for higher resolution assessments. Images for macrophage deposits (¹⁹F-MRI) were overlaid with standard scans of structural anatomy (¹H-MRI) and “heat” maps of the ¹⁹F-signal density constructed. MRI analysis of blasted rats revealed that extensive macrophage infiltration occurred inside the globe of both eyes along with brain accretion mainly in the cerebellum, mid-brain, and surrounding meninges. Ocular macrophage accretion was more robust than that in the brain, consistent with the eyes being externalized; and thus, more susceptible to blast wave damage. Immunohistochemistry (CD68, Iba-1, and GFAP) confirmed that the eyes (retinas) and brain, especially the optic tracts, contain abundant activated macrophages, microglia, and astrocytes. Cell morphology stains (H&E and silver) also showed there to be ongoing neurodegeneration present in the corresponding retinal and brain regions.

SUPPORT: DoD grants from the MOMRP and USAMRMC/CDMRP, #: W81XWH-14-2-0178.

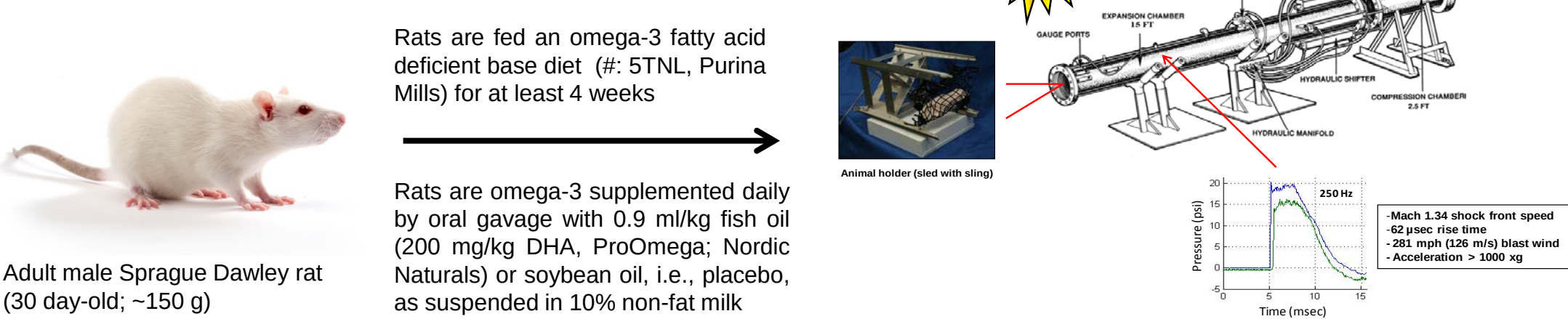
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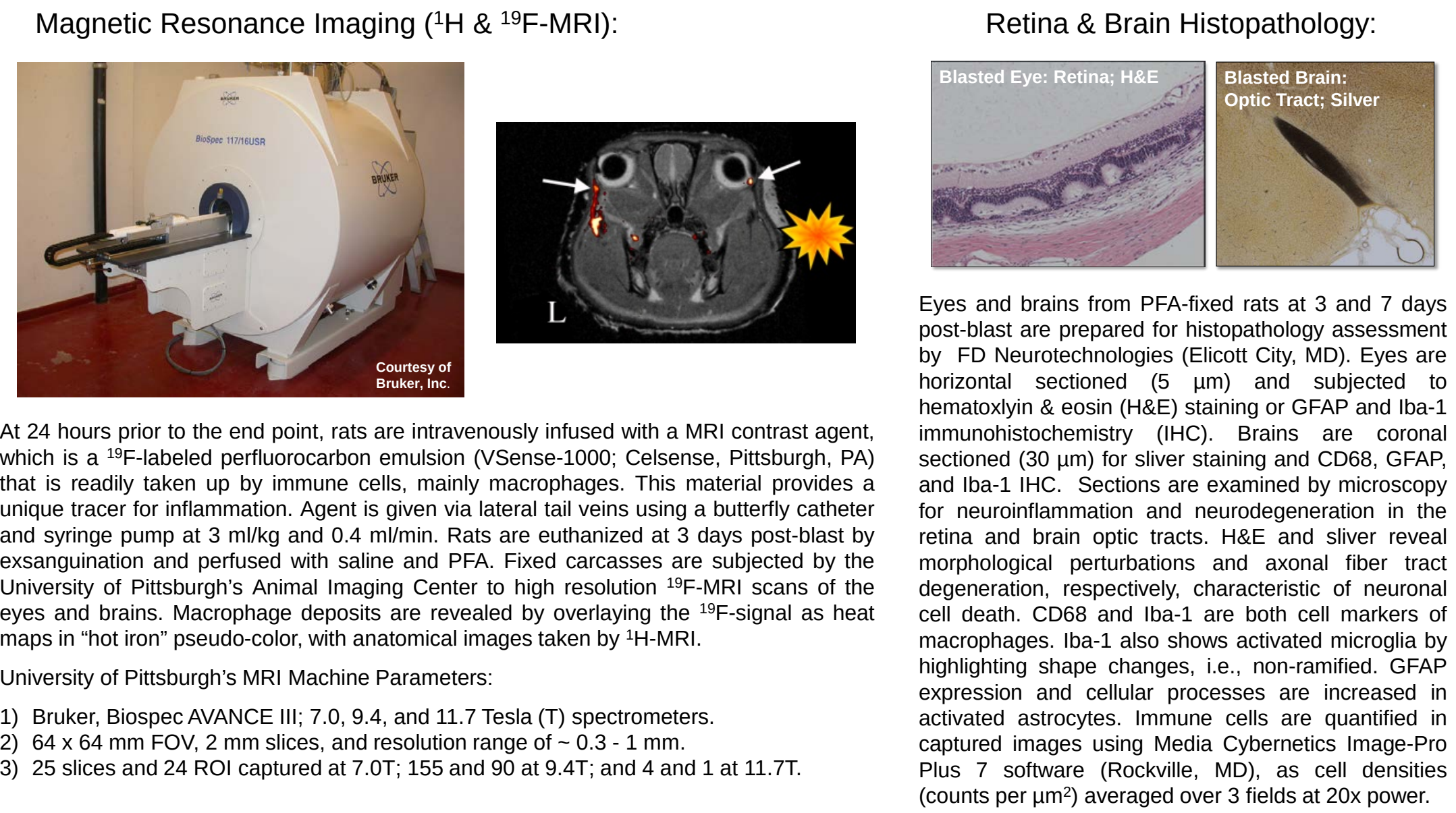
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In our study, rats were maintained on a long chain omega-3 fatty acid deficient or enriched (fish oil) diets and then exposed to high fidelity simulated blast waves in a shock tube. The resulting underlying neuroinflammation and neurodegeneration in the brain and retina was characterized at 3 days post-exposure by Magnetic Resonance Imaging based macrophage tracking (¹⁹F-MRI) and at 3 and 7 days out by histopathology (H&E and silver stains; and CD68, GFAP, and Iba-1 immunohistochemistry).

METHODS



Outcome measures at 3 and 7 days post-double blast:



DATA and RESULTS

Double blast impact on eye and brain macrophage accumulation at 3 days post-exposure; both dietary treatment groups show similar inflammation intensities on ¹⁹F-MRI and ¹H-MRI scans:

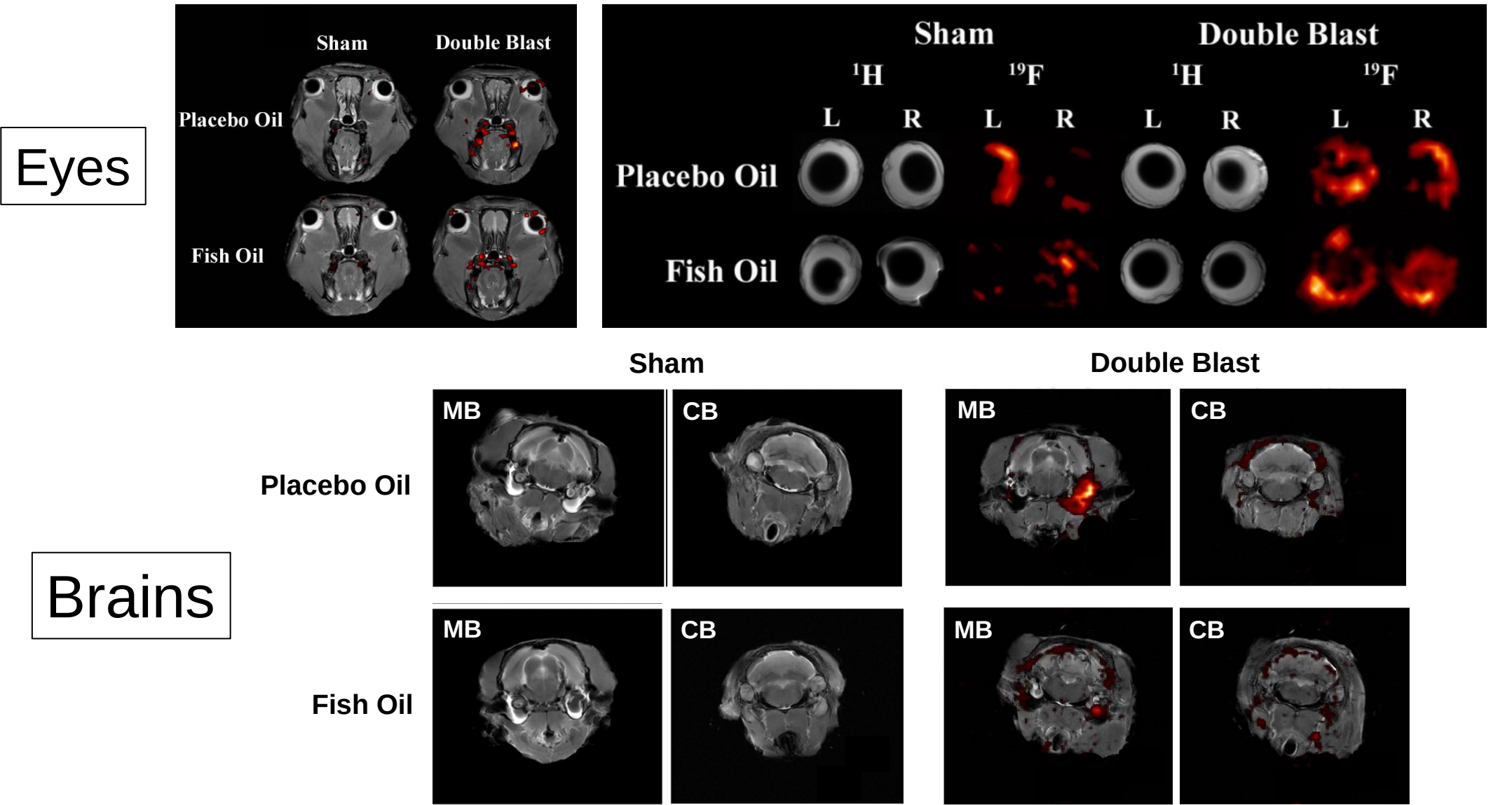


Figure 1. ¹H-MRI anatomical and ¹⁹F-MRI macrophage tracking in rats. In situ images (7.0 & 11.7T) of eyes and brains (cerebellum; CB and mid-brain; MB regions) of representative sham and blasted animals (placebo and fish oil fed). ¹⁹F-signal, as shown in “hot iron” pseudo-color, suggests marked immune cell infiltration is occurring in the blasted eyes and brains, despite dietary treatments. Brain signal is mainly near the meninges and skull bones.

Double blast impact on macrophage accumulation in eyes and related skull bones at 3 days post-exposure; both dietary treatment groups show a similar degree of inflammation:

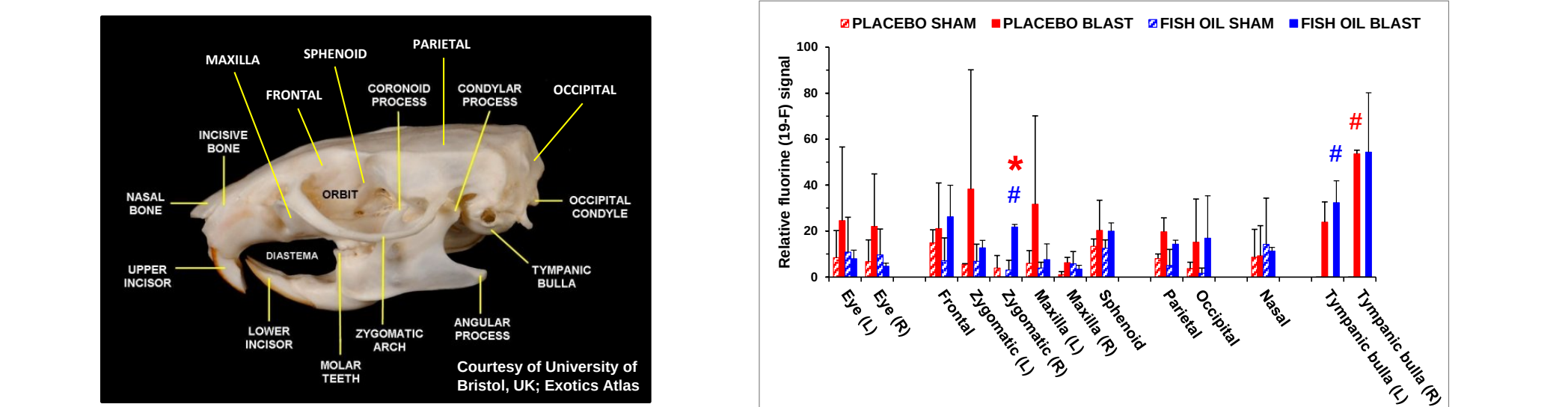


Figure 2. Bar graph for ¹⁹F-MRI signals, as an indication of macrophage accumulation, found in placebo versus fish oil treated rats following double blast exposure or as shams (placebo = red and fish oil = blue, n = 4 each). Diagram is shown for bone structure of the rat skull. *p > 0.05; significant difference between placebo and fish oil treatments alone, as by t-test. # p < 0.05; significant difference between sham and blasted animals.

Double blast impact on eyes at 3 days post-exposure; ¹⁹F-MRI macrophage cell tracking results agree with retina histopathology for neurodegeneration and immune cell infiltration:

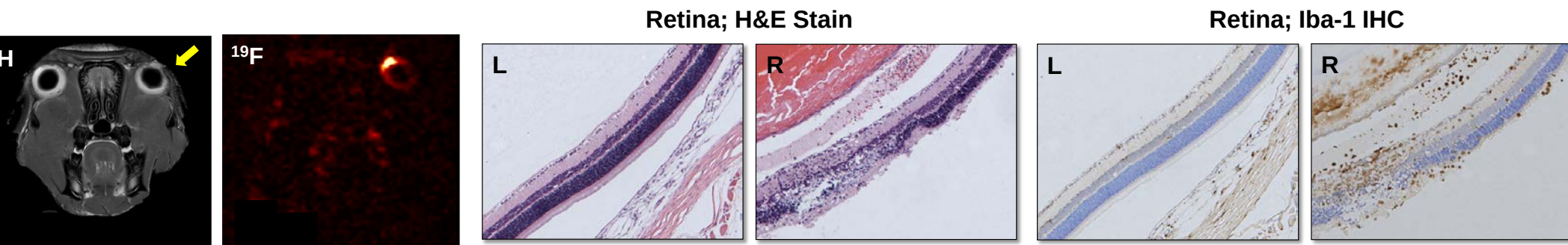


Figure 3. MRI and histopathology of a representative placebo oil treated rat at 3 days post-blast. In situ ¹H and ¹⁹F-MRI (anatomical and macrophage images) of the rat's head is shown at a coronal plane passing through both eyes. Retina histopathology for the corresponding left and right eyes is shown for H&E stain for degenerating neurons (e.g. photoreceptors) and Iba-1 IHC for microglia and macrophages.

Double blast impact on eye (retina) and brain (optic tract) histopathology at 7 days post-exposure; both dietary treatment groups show a similar degree of neurodegeneration and immune cell activity:

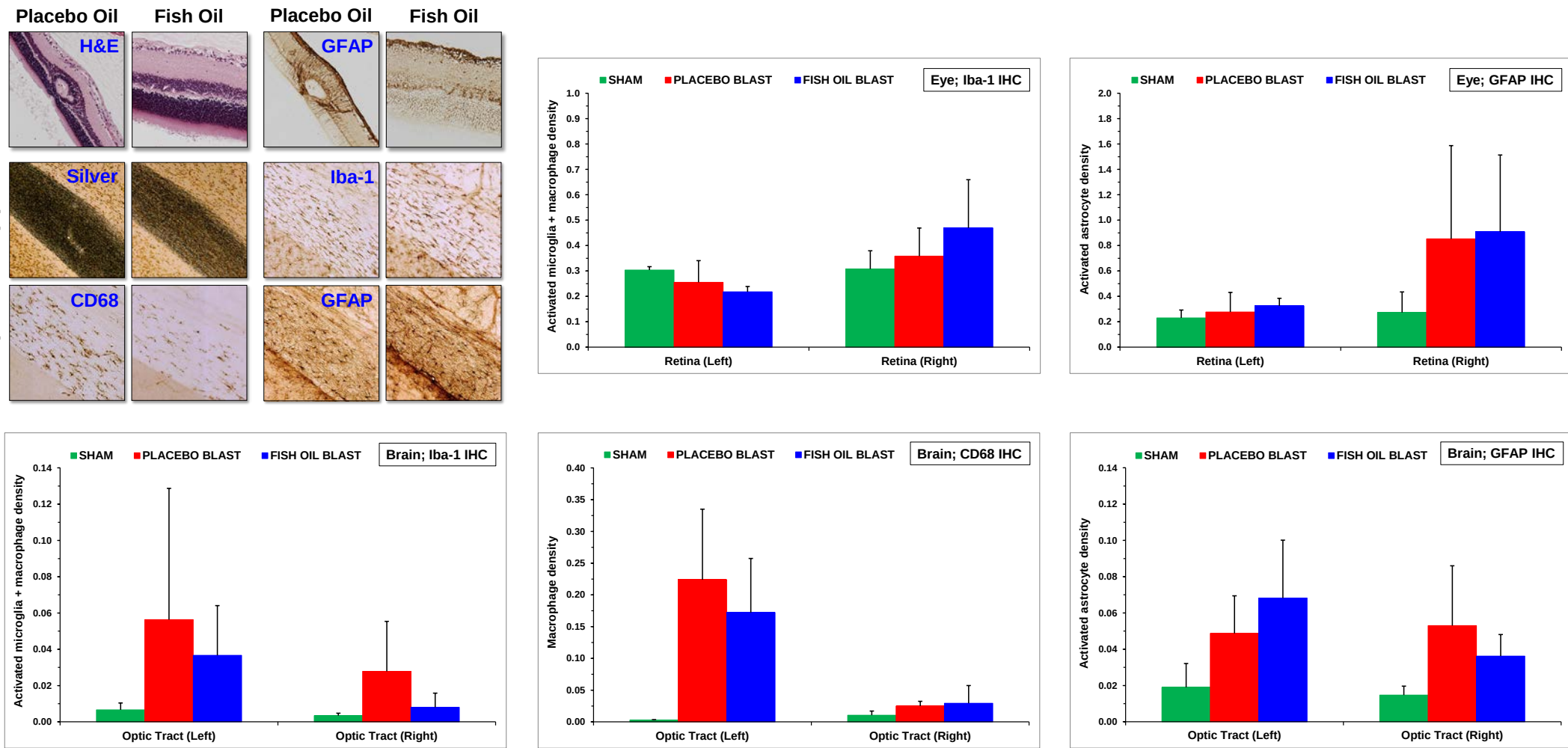


Figure 4. Histopathology of eyes (retina) and brains (optic tract) from representative placebo and fish oil treated rats at 7 days post-blast. Microscope images are shown for retinas and optic tracts under H&E and/or silver stain (degenerating neurons) and Iba-1, CD68, and GFAP IHC (activated microglia + macrophages, macrophages, and activated astrocytes, respectively). Retinal Iba-1 images are not shown here (see Fig. 4), but treatments appeared similar. Bar graphs for immune cell densities (counts per μm²) of retina and optic tracts (left and right) are also given (lumped shams = green, placebo = red, and fish oil = blue; n = 2, 3, 3, and 3, respectively. Current group sizes do not justify statistical comparisons.

CONCLUSIONS

Our current findings with ¹⁹F-MRI macrophage tracking and histopathology in rats reveal that immune cells involved in neurodegeneration and neuroinflammation processes, i.e., macrophages, microglia, and astrocytes, have accumulated in the retina and brain by 7 days following exposure to blast waves. Dietary omega-3 fatty acids, as given by high dose fish oil, have so far shown little, if any, ability to alleviate these injury events, despite having potent anti-inflammatory properties targeting immune cell activities. Reason for lack of a robust treatment effect is unclear, but may be reflective of the injury time frame examined i.e., acute phase. Thus, we plan to extend evaluations over more prolonged post-blast intervals.

Characterization of Inflammation Processes in the Visual System of Rats Induced by Exposure to Primary Blast Waves

¹James DeMar, ¹John Rosenberger, ¹Andrew Batuure, ¹Daniel Thadeio, ¹Donna Wilder, ¹Meghan Mccuiston, ²Patrick Kochanek, ³Lesley Foley, ³Kevin Hitchens, and ¹Joseph Long.

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Blast injury is arguably the greatest threat to Warfighters in current campaigns, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain from blast shock waves. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast, which allows evaluations of neuronal injuries to the retina and brain. Our hypothesis is that immune cells play a primary role in exacerbating neurodegeneration following blast. Our objective is to monitor the nature and timing of neuroinflammation processes so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutrients known to be anti-inflammatory, e.g. omega-3 polyunsaturated fatty acids, on blast vulnerability. Adult male rats were fed for one month an omega-3 fatty acid deficient versus enriched (fish oil supplemented) diet. Blast injury was produced in anesthetized animals secured in a compressed air driven shock tube and then exposed twice (1 min interval) to blast over pressure waves (20 psi, 8 msec). At 3 to 28 days post-blast, retina and brain injuries were followed by electroretinography, visual acuity assessment, magnetic resonance imaging, histopathology, and cytokine level outcome measures. Our findings reveal that vision impairments occur early post-blast (i.e., within 7 days), and are accompanied by neurodegeneration in the retina and brain along with macrophage accretion, activated microglia and astrocytes, and increased cytokines. Dietary omega-3 fatty acids have so far shown slight, if any, ability to alleviate these acute injury events. Overall, our mission is to discover new drug treatments for blast-induced neurotrauma sustained by military personnel.

Supported by DoD grants from MOMRP and USAMRMC / CDMRP, #: W81XWH-14-2-0178.

the retina and brain. Thus, there is an urgent need to do advanced studies in a well-established rodent model of blast. Our hypothesis is that immune cell mediated processes play a primary role in dictating the extent of neuronal cell death in retina and brain following blast. Using adult rats exposed to shock tube generated blast waves, our objective is to longitudinally monitor up to 28 days the nature and timing of immune cell guided inflammatory processes in the injured retina and brain as to discern potential drug targets and therapeutic windows. We are also examining the impact of interventions known to be anti-inflammatory, i.e., giving the rats a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Overall, our mission is to provide data that will ultimately lead to discovery of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: DoD grant awards from the MOMRP and USAMRMC / CDMRP/ Vision Research Program #: W81XWH-14-2-0178.

Introduction

- ❑ Blast injuries have resulted since 2008 in more than 280,000 cases of traumatic brain injury in U.S. Soldiers, with frequent symptoms of vision loss (Capo-Aponte, 2012; Lemke, 2013).
- ❑ While brain visual centers can be affected, 43% display closed-eye injuries with 26% having retina damage (Cockerham, 2011).
- ❑ Soldiers are issued protective goggles but still suffer eye injures, such as from blast wave penetration (Weichel, 2008).
- ❑ Few animal studies have tried to characterize visual system injuries as generated by rigorously simulated blast waves; and only three have evaluated drug treatments (review by DeMar, 2016).
- ❑ Following blast wave injury, the brain and retina can undergo acute inflammation accompanied by immune cell activation, cytokine release, and neuronal cell degeneration (Serhan, 2010).
- ❑ Metabolites of dietary omega-3 polyunsaturated fatty acids can suppress immune cell activation (Serhan, 2008, 2011, 2012).

References:
Capó-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
DeMar et al., 2016; Front. Neurol. 7: 59(1-12).
Lemke et al., 2013; JAMA Ophthalmol. 131(12): 1602-1609.
Serhan et al., 2008, 2010, 2011, & 2012; British J. Pharm. 153: 5200-5215, Am. J. Pathol. 177(4) 1576-1591, Curr. Top. Med. Chem. 11(6) 629-647, & Chem. Rev. 111(10) 5922-5943.
Weichel et al., 2008; Curr. Opin. Ophthalmol. 19(6): 519-525.

Aim of Study

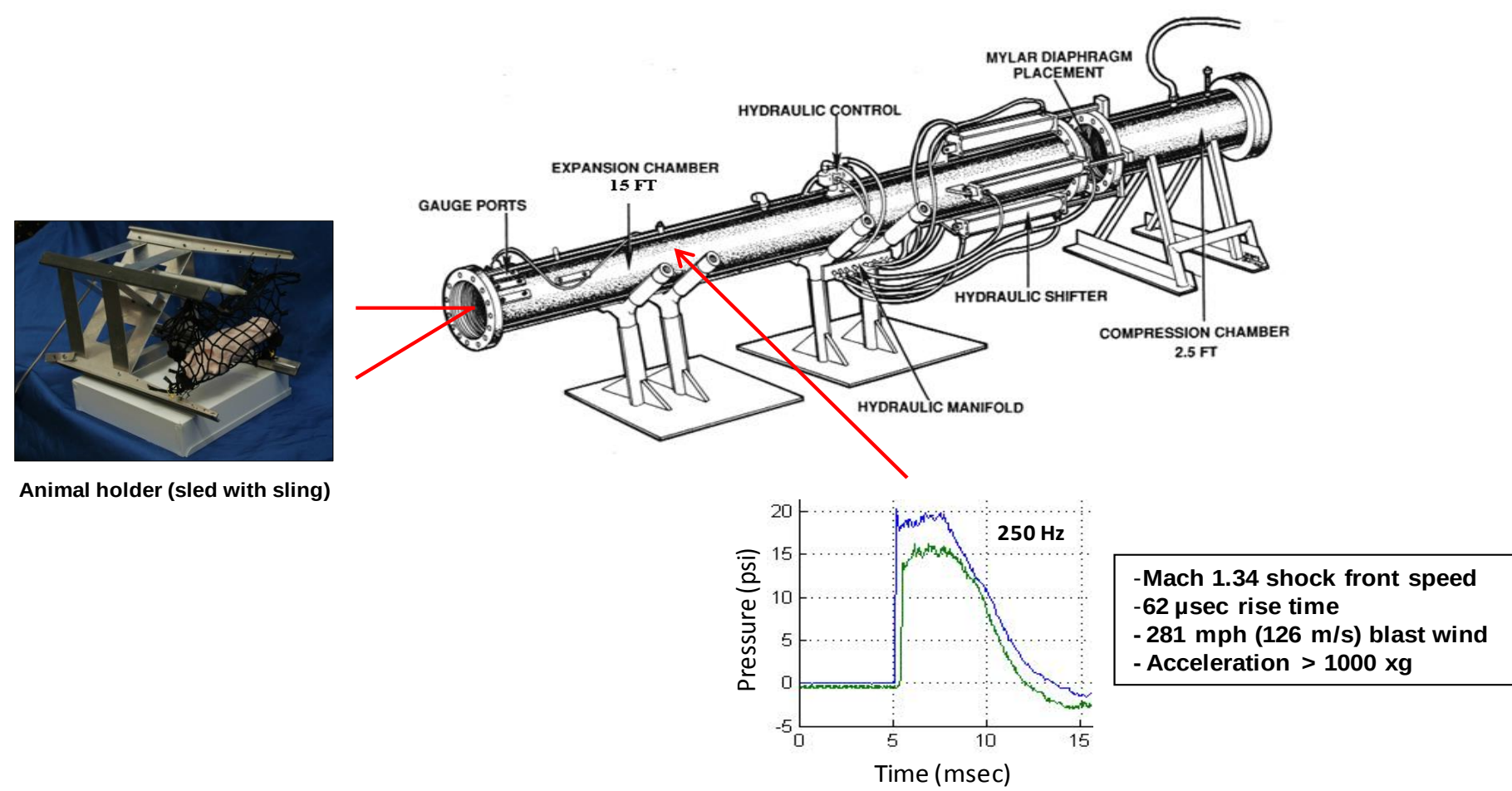
In rats raised on omega-3 fatty acid deficient or enriched diets and then exposed to high fidelity simulated blast waves, characterize up to 28 days out the visual dysfunction and underlying neuro-inflammation / degeneration due to retina and brain injures, as by:

- 1) Visual acuity (optokinetics) and electroretinography (ERG).
- 2) Bloodwork, tissue fatty acids and cytokines, and Magnetic Resonance Imaging (MRI) based immune cell tracking.
- 3) Histopathology (H&E and silver stains; and GFAP & Iba-1 IHC).

are given an equal volume of soybean oil.

Simulation of Primary Blast Wave Injuries:

❑ Rats are exposed under isoflurane anesthesia to two blast over pressure waves, i.e., double blast (20 psi; 1min interval), in a right-side on position, using a compressed air driven shock tube.



Visual Acuity (Optokinetics):

Using an optokinetics device (Optomotry; Cerebral-Mechanics), rats are put on a pedestal in a chamber, where a rotating bar pattern is shown on four LCD monitors. Rotation speed is increased stepwise to narrow the bar's width; and visual acuity threshold (cycles/degree) is found, when animal ceases head-eye tracking movements (nystagmus). Separate eye acuities are determined by driving the rotation in opposite directions.

Electroretinography (ERG):

Rats are dark adapted for 16 h. Using a full field flash Ganzfeld ERG device (Color Dome; Diagnosys), rats are placed under isoflurane, pupils are drug-dilated, and electrodes are put on eyes (recording), cheeks (reference), and tail (ground). Eyes are flashed with light (10 cd.s/m²; 5 msec); and evoked retina photoreceptor potentials are recorded (a-waveform).

Bloodwork, Tissue Fatty Acids and Cytokines, and ¹⁹F-MRI Based Immune Cell Tracking:

Blood glucose is determined on living rats by tail stick and a handheld test-strip monitor (Contour; Bayer). Terminal rat blood (cardiac puncture) is submitted to WRAIR Clinical Pathology for Complete Blood Count (CBC) and chemistry panel. Total fatty acid profiles of fresh liver are obtained, by lipid extraction and GC/MS (7890A/5975C; Agilent) methods. Cytokine levels of fresh brain are found, using protein lysates and immunoassay arrays (rat 10-plex; Luminex, R&D Systems). Macrophage immune cell deposits are revealed in eyes and brains of PFA-fixed rats, using a pre-injected (24 h) ¹⁹F-contrast agent (V-Sense; Celsense) and high resolution MRI scanning (Univ. of Pittsburgh).

Histopathology (H&E, Silver, Iba-1, and GFAP):

Following transcordial perfusion of rats with PFA, fixed eyes and brains are processed (FD Neurotechnologies) into sections and then H&E (eyes), silver (brains), GFAP (both) and Iba-1(both) stained / immunohistochemistry (IHC) slides; which are examined by microscopy for signs of immune cell infiltration and neurodegeneration.

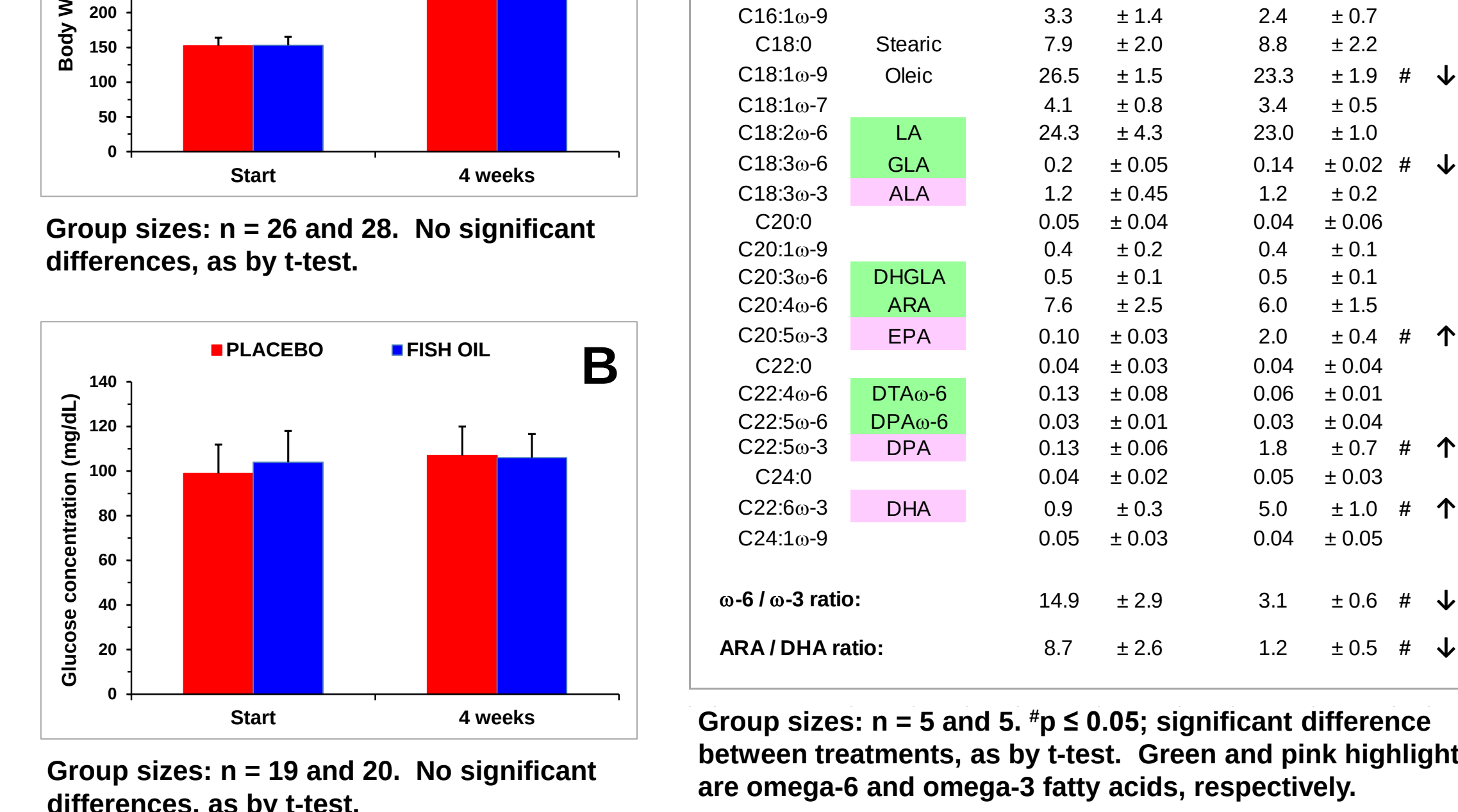


Figure 1. Body weights (A), blood glucose (B), and liver fatty acids (C) of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets for 4 weeks.

Double Blast Impact on Survival, Righting Reflex, and Bloodwork:

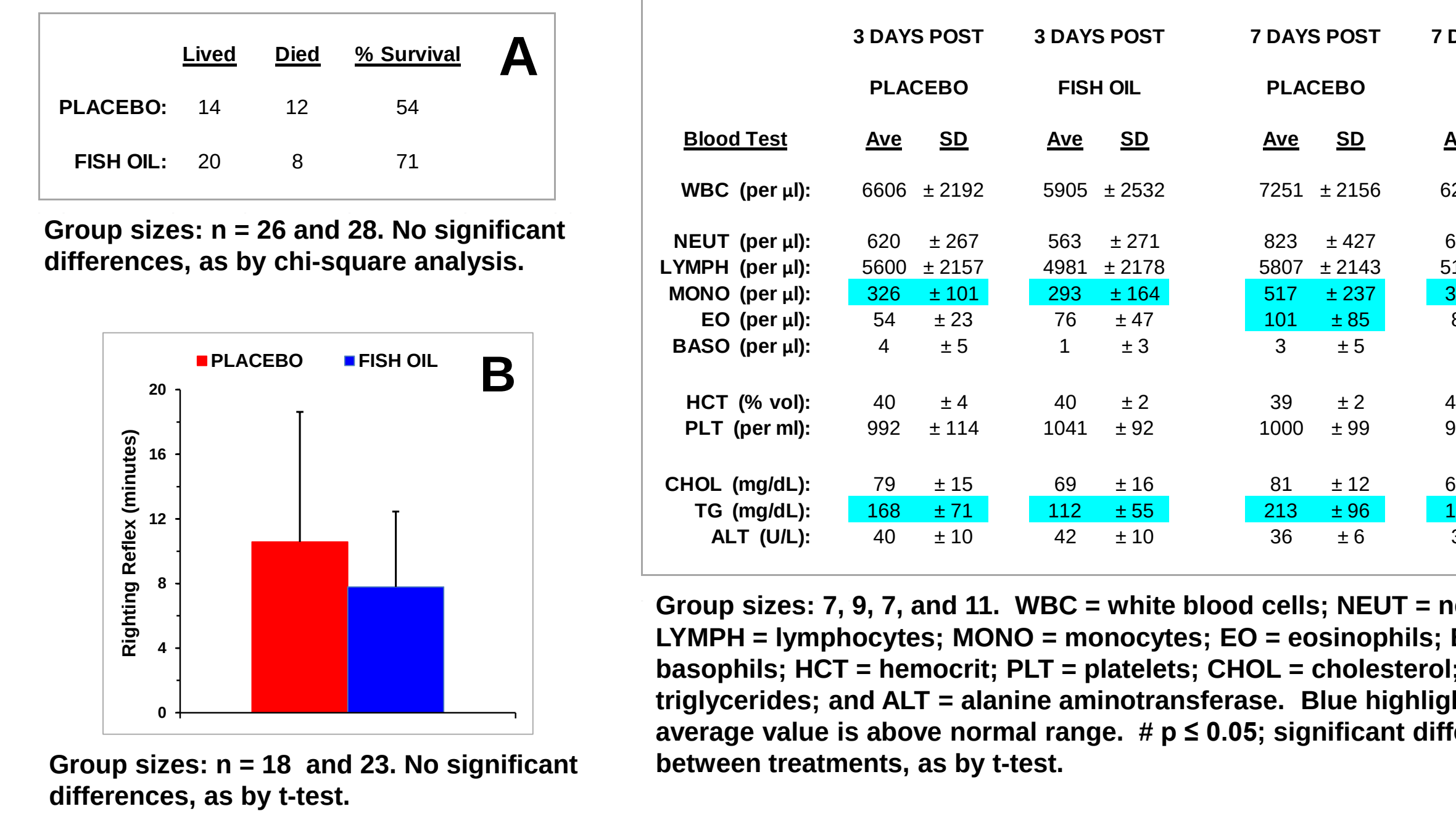


Figure 2. Percent survival (A), righting reflex (B), and bloodwork (C) of rats fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets immediately after blasting and at 3 and 7 days post-blast.

Double Blast Impact on Visual Acuities and Electroretinography (ERG):

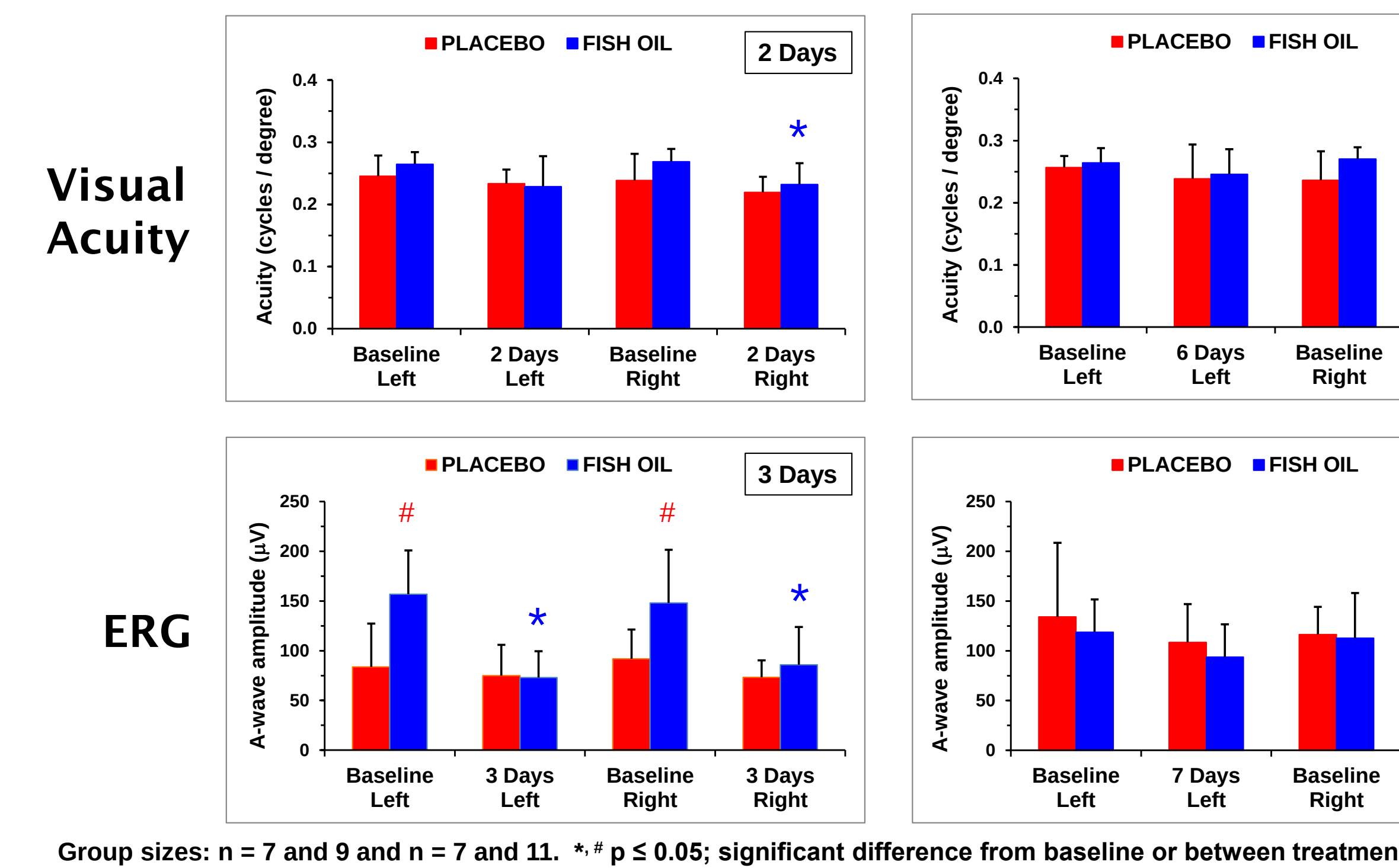


Figure 3. Visual acuities (cycles/degree) and ERG amplitudes (μV) of rats (left and right eyes) fed omega-3 fatty acid deficient (placebo) and enriched (fish oil) diets at baseline and 2 - 3 and 6 - 7 days post-blast.

Magnetic Resonance Imaging (¹⁹F-MRI) based Tracking of Macrophage Infiltration in the Visual System of Rats following Exposure to Primary Blast Waves

¹James C. DeMar, ¹John G. Rosenberger, ¹Andrew B. Batuure, ¹Donna M. Wilder, ¹Meghan A. Mccuiston, ³Lesley M. Foley, ²Patrick M. Kochanek, and ³T. Kevin Hitchens, and ¹Joseph B. Long.

¹Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience Research, Walter Reed Army Institute of Research, Silver Spring, MD 20910; ²Safar Center for Resuscitation Research and ³Animal Imaging Center, University of Pittsburgh, Pittsburgh, PA 15261.

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual center from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast injury. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast exposure. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, e.g. a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Adult male rats were raised for one month on an omega-3 fatty acid-deficient chow ("Typical American diet"; Test Diet, Purina Mills) versus one that was enriched by daily oral supplementation with ocean fish oil. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). Shams were also placed on the diets, but received only anesthesia exposure. At an acute stage post-blast, i.e., 3 days, the rats (n = 8 per treatment) were euthanized and paraformaldehyde perfused. Migration of macrophages to retina and brain injury sites was tracked in situ by magnetic resonance imaging (MRI) of the carcasses, using a ¹⁹F-labeled, i.e., perfluorocarbon, contrast agent (VS1000; Celsense) that was selectively taken up by macrophages following intravenous administration 24 hours before the end point. Animals were whole body imaged on a 9.4T spectrometer (AVIII HD, Bruker Biospin) and then the excised eyes and brains reimaged at 11.7T for higher resolution assessments. Images for macrophage deposits (¹⁹F-MRI) were overlaid with standard scans of structural anatomy (¹H-MRI) and signal density "heat" maps constructed. MRI analysis of blasted rats revealed that extensive macrophage infiltration occurred inside the globe of both eyes along with brain accretion mainly in the cerebellum, mid-brain, and surrounding meninges. Ocular macrophage accretion was more robust than that in the brain, consistent with the eyes being externalized; and thus, more susceptible to blast wave damage. Immunohistochemistry (CD68, Iba-1, and GFAP) confirmed that the eyes (retinas) and brain, especially the optic tracts, contain abundant activated macrophages, microglia, and astrocytes. Cell morphology stains (H&E and silver) also showed

there to be ongoing neurodegeneration present in the corresponding retinal and brain regions. Dietary omega-3 fatty acids, however, have shown so far a very modest ability, if any, to alleviate the majority of these acute neuropathological events. Thus, we plan to extend our study to chronic time points post-blast, i.e., up to 28 days post-injury, to look for a delayed therapeutic effect of omega-3 supplementation in promoting resiliency of neural tissues towards blast exposure. Overall, our mission is to provide data that will ultimately lead to discovery and animal testing of new drug treatments for blast-induced neurotrauma sustained to by members of the US Army.

SUPPORT: This work is supported by DoD grant awards from the MOMRP and USAMRMC / CDMRP (#: W81XWH-14-2-0178).



ABSTRACT

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes (retina) or brain visual center from blast shock waves. Despite the difficult life-long disability that permanent loss of vision represents, there are very few animal studies that have rigorously assessed blast wave injuries to the visual system. Many of these studies suffer from poor simulation of blast wave injuries or are limited in their biological outcome measures. None have studied the interplay of blast damage to the retina and brain. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast injury. Our hypothesis is that immune cell mediated processes play a primary role dictating the extent of neuronal cell death in retina and brain visual centers following blast exposure. Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes in the retina and brain after blast so as to discern potential drug targets and therapeutic windows. We are also examining the impact of nutritional treatments known to be anti-inflammatory, e.g. a diet enriched in omega-3 polyunsaturated fatty acids, on blast injury vulnerability. Adult male rats were raised for one month on an omega-3 fatty acid-deficient chow (“Typical American diet”; Test Diet, Purina Mills) versus one that was enriched by daily oral supplementation with ocean fish oil. Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). Shams were also placed on the diets, but received only anesthesia exposure. At an acute stage post-blast, i.e., 3 days, the rats (n = 8 per treatment) were euthanized by exsanguination and perfused with saline and paraformaldehyde. Migration of macrophages to retina and brain injury sites was tracked in situ by magnetic resonance imaging (MRI) of the carcasses, using a ¹⁹F-labeled, i.e., perfluorocarbon, contrast agent (VS1000; Celsense) that was selectively taken up by macrophages following intravenous administration 24 hours before the end point. Animals were whole body imaged on a 9.4T spectrometer (Biospec AVANCE III, Bruker) and then the excised eyes and brains reimaged at 11.7T for higher resolution assessments. Images for macrophage deposits (¹⁹F-MRI) were overlaid with standard scans of structural anatomy (¹H-MRI) and “heat” maps of the ¹⁹F-signal density constructed. MRI analysis of blasted rats revealed that extensive macrophage infiltration occurred inside the globe of both eyes along with brain accretion mainly in the cerebellum, mid-brain, and surrounding meninges. Ocular macrophage accretion was more robust than that in the brain, consistent with the eyes being externalized; and thus, more susceptible to blast wave damage. Immunohistochemistry (CD68, Iba-1, and GFAP) confirmed that the eyes (retinas) and brain, especially the optic tracts, contain abundant activated macrophages, microglia, and astrocytes. Cell morphology stains (H&E and silver) also showed there to be ongoing neurodegeneration present in the corresponding retinal and brain regions.

SUPPORT: DoD grants from the MOMRP and USAMRMC/CDMRP, #: W81XWH-14-2-0178.

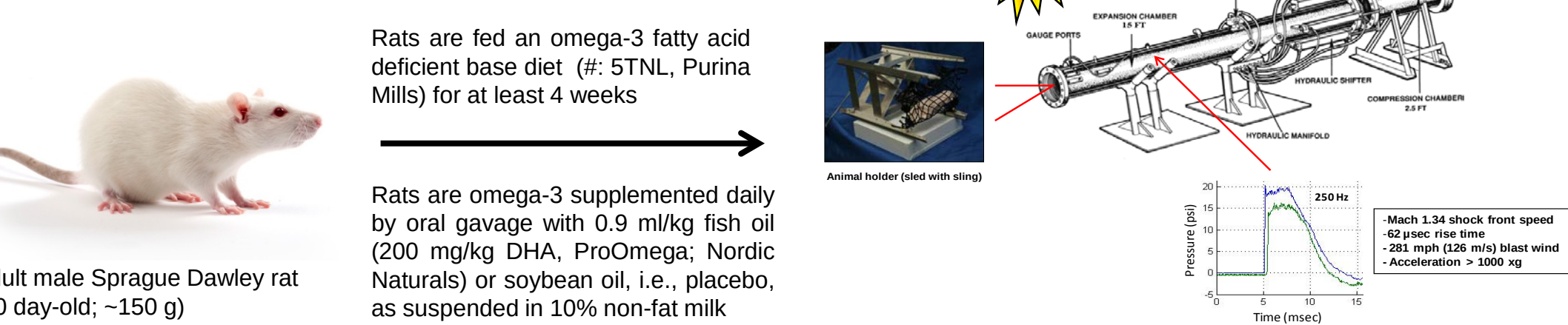
BACKGROUND

Blast exposure has caused since 2008 about 280,000 cases of traumatic brain injury in U.S. Soldiers, often with accompanying vision loss symptoms (Capo-Aponte, 2012; Lemke, 2013). Of these patients, 43% display closed-eye injuries with 26% having retina damage (Cockerham, 2011). While soldiers wear protective goggles, they still can suffer eye injuries, e.g. blast wave penetration (Weichel, 2008). Few animal studies have tried to characterize visual system injuries as generated by high fidelity blast waves and/or have evaluated drug treatments (review by DeMar, 2016). Following blast wave injury, the brain and retina can undergo acute inflammation accompanied by immune cell activation, cytokine release, and neuronal cell degeneration (Serhan, 2010). Dietary omega-3 polyunsaturated fatty acids are converted to molecules, e.g. neuroprotectins and resolvins, which can suppress immune cell activation and thus, may represent a practical therapeutic intervention (Serhan, 2008, 2011, 2012).

References: Capo-Aponte, 2012, Mil. Med. 177: 804-813; Cockerham, 2011, N. Engl. J. Med. 364: 2172-2173; DeMar, 2016, Front. Neurol. 7: 59 (1-12); Lemke, 2013, JAMA Ophthalmol. 131: 1602-1609; and Serhan, 2008, 2010, 2011, & 2012, British J. Pharm. 153: 5200-5215, Am. J. Pathol. 177: 1576-1591, Curr. Top. Med. Chem. 11: 629-647, & Chem. Rev. 111(10) 5922-5943; and Weichel, 2008, Curr. Opin. Ophthalmol. 19: 519-525.

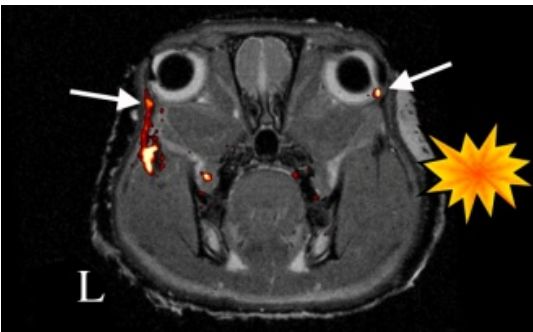
In our study, rats were maintained on a long chain omega-3 fatty acid deficient or enriched (fish oil) diets and then exposed to high fidelity simulated blast waves in a shock tube. The resulting underlying neuroinflammation and neurodegeneration in the brain and retina was characterized at 3 days post-exposure by Magnetic Resonance Imaging based macrophage tracking (¹⁹F-MRI) and at 3 and 7 days out by histopathology (H&E and silver stains; and CD68, GFAP, and Iba-1 immunohistochemistry).

METHODS

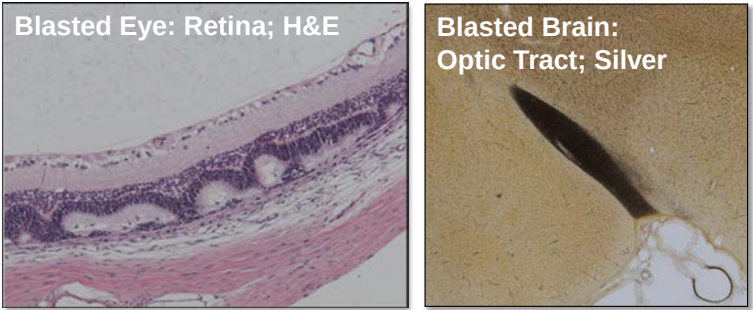


Outcome measures at 3 and 7 days post-double blast:

Magnetic Resonance Imaging (¹H & ¹⁹F-MRI):



Retina & Brain Histopathology:



At 24 hours prior to the end point, rats are intravenously infused with a MRI contrast agent, which is a ¹⁹F-labeled perfluorocarbon emulsion (VSense-1000; Celsense, Pittsburgh, PA) that is readily taken up by immune cells, mainly macrophages. This material provides a unique tracer for inflammation. Agent is given via lateral tail veins using a butterfly catheter and syringe pump at 3 ml/kg and 0.4 ml/min. Rats are euthanized at 3 days post-blast by exsanguination and perfused with saline and PFA. Fixed carcasses are subjected by the University of Pittsburgh's Animal Imaging Center to high resolution ¹⁹F-MRI scans of the eyes and brains. Macrophage deposits are revealed by overlaying the ¹⁹F-signal as heat maps in “hot iron” pseudo-color, with anatomical images taken by ¹H-MRI.

University of Pittsburgh's MRI Machine Parameters:

- 1) Bruker, Biospec AVANCE III; 7.0, 9.4, and 11.7 Tesla (T) spectrometers.
- 2) 64 x 64 mm FOV, 2 mm slices, and resolution range of ~ 0.3 - 1 mm.
- 3) 25 slices and 24 ROI captured at 7.0T; 155 and 90 at 9.4T; and 4 and 1 at 11.7T.

DATA and RESULTS

Double blast impact on eye and brain macrophage accumulation at 3 days post-exposure; both dietary treatment groups show similar inflammation intensities on ¹⁹F-MRI and ¹H-MRI scans:

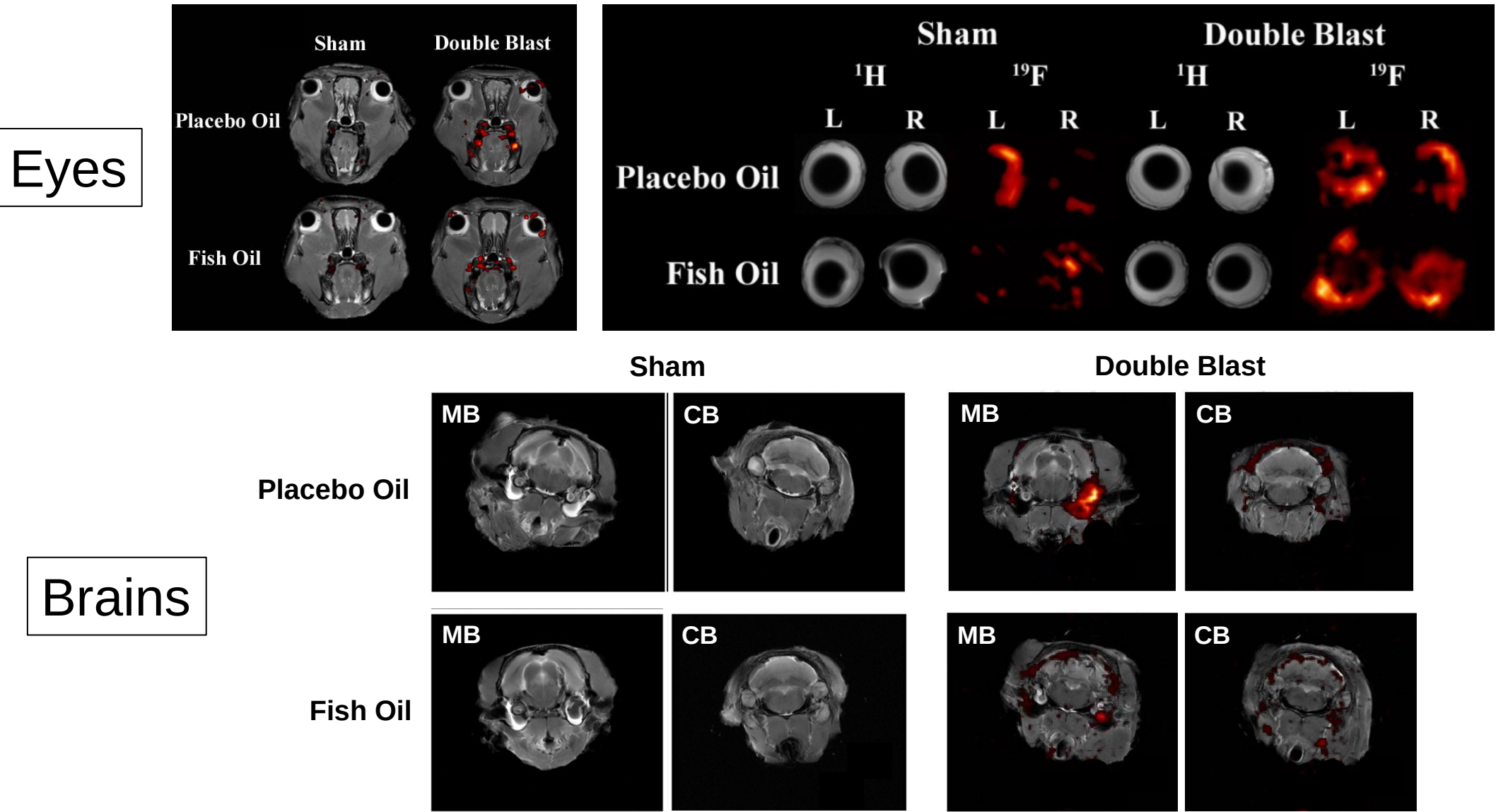


Figure 1. ¹H-MRI anatomical and ¹⁹F-MRI macrophage tracking in rats. In situ images (7.0 & 11.7T) of eyes and brains (cerebellum; CB and mid-brain; MB regions) of representative sham and blasted animals (placebo and fish oil fed). ¹⁹F-signal, as shown in “hot iron” pseudo-color, suggests marked immune cell infiltration is occurring in the blasted eyes and brains, despite dietary treatments. Brain signal is mainly near the meninges and skull bones.

Double blast impact on macrophage accumulation in eyes and related skull bones at 3 days post-exposure; both dietary treatment groups show a similar degree of inflammation:

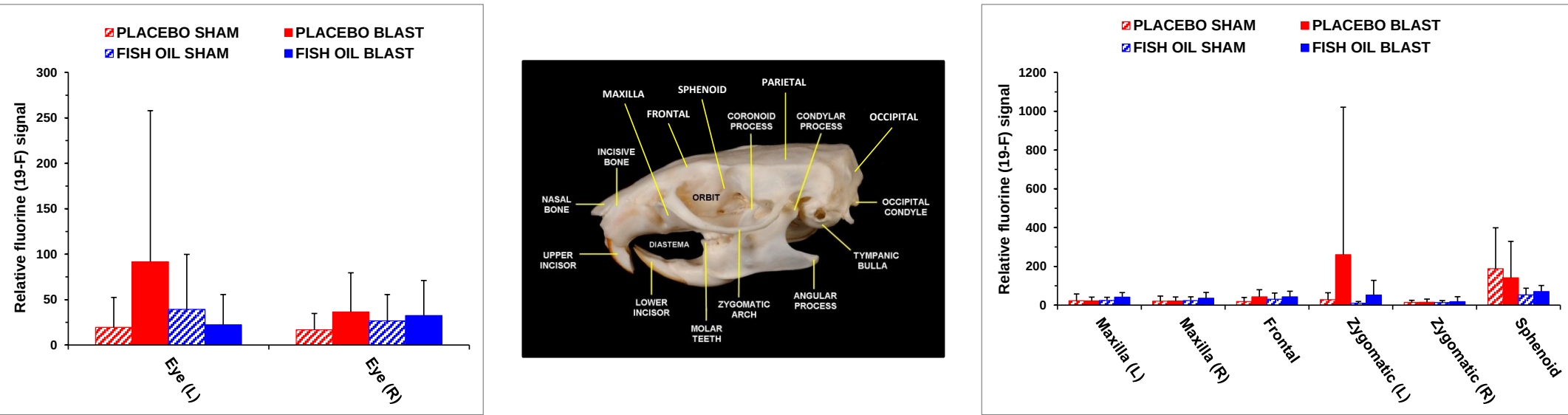


Figure 2. Bar graph for ¹⁹F-MRI signals, as an indication of macrophage accumulation, found in placebo versus fish oil treated rats following double blast exposure or as shams (placebo = red and fish oil = blue, n = 7, 10, 8, and 12). Separate graphs are shown for eyes and the bones making up the surrounding orbital socket. Diagram is shown for bones of the rat skull. No significant differences were detected between the treatment groups.

Double blast impact on eyes at 3 days post-exposure; ¹⁹F-MRI macrophage cell tracking results agree with retina histopathology for neurodegeneration and immune cell infiltration:

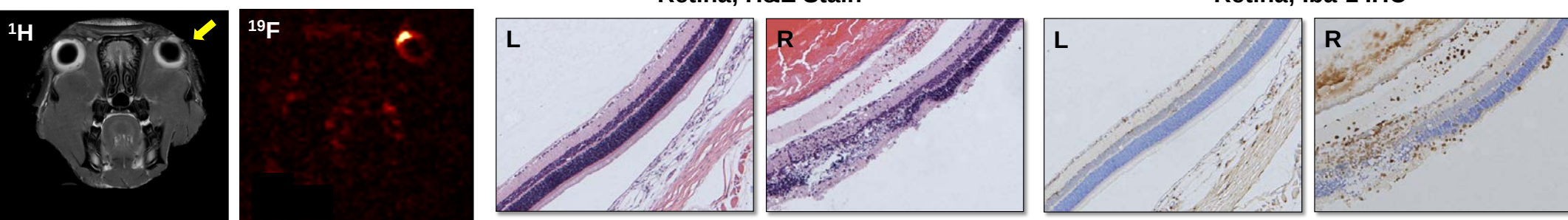


Figure 3. MRI and histopathology of a representative placebo oil treated rat at 3 days post-blast. In situ ¹H and ¹⁹F-MRI (anatomical and macrophage images) of the rat's head is shown at a coronal plane passing through both eyes. Retina histopathology for the corresponding left and right eyes is shown for H&E stain for degenerating neurons (e.g. photoreceptors) and Iba-1 IHC for microglia and macrophages.

Double blast impact on eye (retina) and brain (optic tract) histopathology at 7 days post-exposure; both dietary treatment groups show a similar degree of neurodegeneration and immune cell activity:

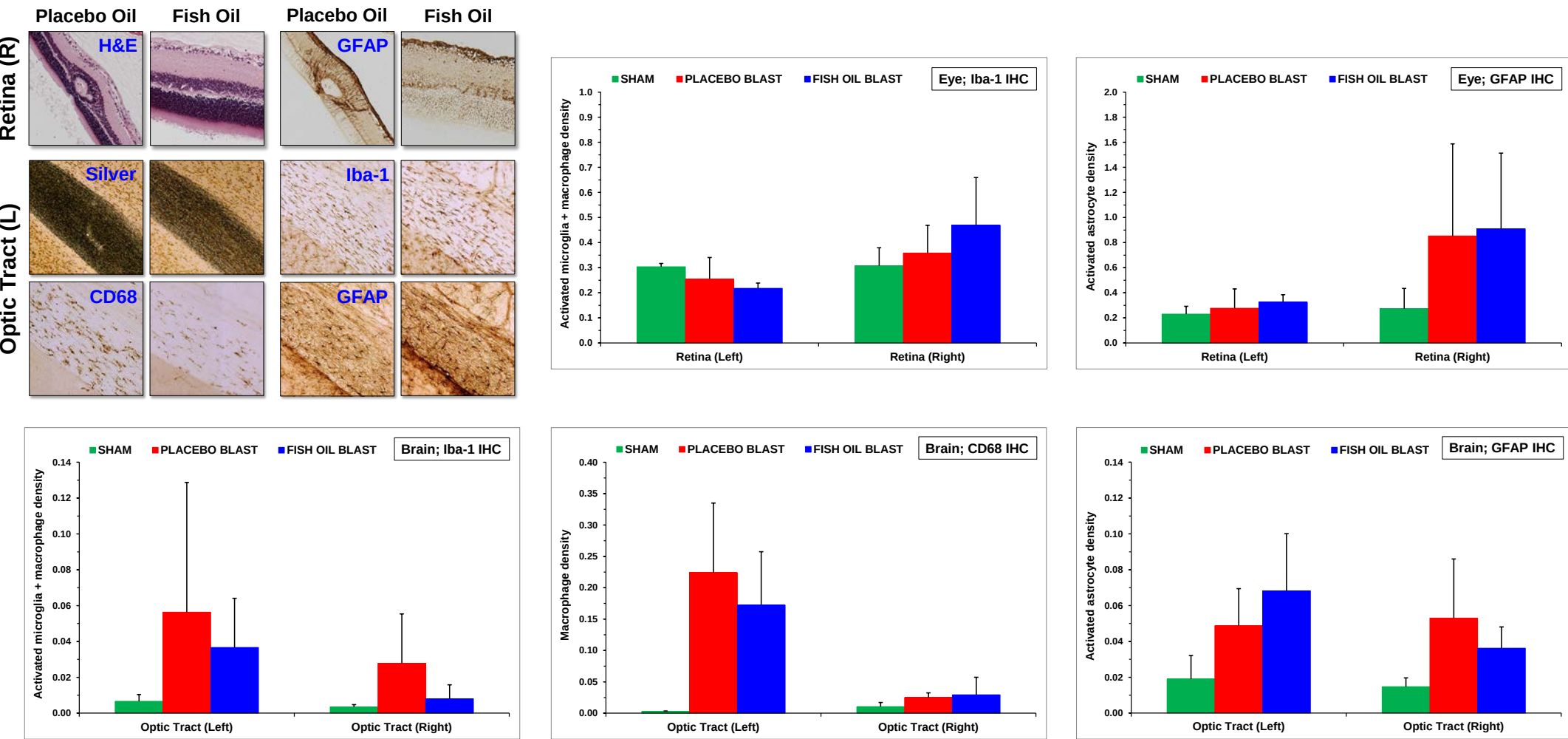


Figure 4. Histopathology of eyes (retina) and brains (optic tract) from representative placebo and fish oil treated rats at 7 days post-blast. Microscope images are shown for retinas and optic tracts under H&E and/or silver stain (degenerating neurons) and Iba-1, CD68, and GFAP IHC (activated microglia + macrophages, macrophages, and activated astrocytes, respectively). Retinal Iba-1 images are not shown here (see Fig. 4), but treatments appeared similar. Bar graphs for immune cell densities (counts per μm²) of retina and optic tracts (left and right) are also given (lumped shams = green, placebo = red, and fish oil = blue; n = 2, 3, 3, and 3, respectively. Current group sizes do not justify statistical comparisons.

CONCLUSIONS

Our current findings with ¹⁹F-MRI macrophage tracking and histopathology in rats reveal that immune cells involved in neurodegeneration and neuroinflammation processes, i.e., macrophages, microglia, and astrocytes, have accumulated in the retina and brain by 7 days following exposure to blast waves. Dietary omega-3 fatty acids, as given by high dose fish oil, have so far shown little, if any, ability to alleviate these injury events, despite having potent anti-inflammatory properties targeting immune cell activities. Reason for lack of a robust treatment effect is unclear, but may be reflective of the injury time frame examined i.e., acute phase. Thus, we plan to extend evaluations over more prolonged post-blast intervals.

Characterization of Inflammation Induced by Exposure to Primary Blast Waves in Rats using ^{19}F MRI

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¹Animal Imaging Center, University of Pittsburgh, Pittsburgh, PA, United States, ²Center for Military Psychiatry and Neuroscience Research, Walter Reed Army Institute of Research, Silver Springs, MD, United States, ³Safar Center for Resuscitation Research, University of Pittsburgh, Pittsburgh, PA, United States, ⁴Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA, United States

Synopsis

Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes following multiple blasts in an animal model, in an effort to discern potential therapeutic windows. Blast, results in a heterogeneous whole-body inflammatory response that can be observed with a perfluorocarbon contrast agent and ^{19}F MRI. A second closely timed blast increased the amount of fluorine signal in injured rats, which most likely represents increased macrophage accumulation. Despite having potent anti-inflammatory properties, we did not observe that a diet rich in omega-3 fatty acids has a significant impact on the inflammatory response to blast injuries.

Introduction

Blast wave injuries caused primarily by improvised explosive devices, have emerged as a leading cause of morbidity and mortality in wars of the twenty-first century¹. Despite the possibility of life-long disability, there are very few animal studies that have rigorously assessed blast wave injuries, especially to the visual system. Thus, there is an urgent need to carry out advanced studies in a well-established rodent model of blast².

Our objective is to longitudinally monitor the nature and timing of immune cell guided inflammatory processes after blast so as to discern potential drug targets and therapeutic windows. This was done using a perfluorocarbon-based cell tracking agent (VS1000, Celsense, Pittsburgh, PA). With this tracer agent it is possible to label immune cells, mainly macrophages, *in situ*, and noninvasively detect the accumulation of these labeled immune cells *in vivo*. We also examined the impact of nutritional treatments known to be anti-inflammatory (e.g. a diet enriched in omega-3 polyunsaturated fatty acids) on blast injury vulnerability.

Methods

Adult male rats were maintained for one month on omega-3 fatty acid-enriched (via fish oil supplementation) versus -deficient diets (a Placebo Oil). Blast injury was produced in anesthetized rats secured in a compressed air driven shock tube and then exposed to two closely-coupled repeated blast over pressure waves (20 psi total pressure, 8 msec duration, 1 min interval). At 24 hours post blast, animals were injected with a perfluorocarbon contrast agent (VS1000, Celsense, Pittsburgh, PA) then euthanized and perfused 48 hours later.

Fixed animals were imaged on a 9.4T AVIII HD spectrometer (Bruker Biospin, Billerica, MA) equipped with a double tuned 72 mm $^1\text{H}/^{19}\text{F}$ coil. A ^1H and ^{19}F T₁-

RARE sequence was performed using the following parameters: TE/TR = 7/4000 ms, RARE factor = 8, FOV = 64 x 64 mm, matrix = 256 x 256 (^1H) and 64 x 64 (^{19}F). Following whole animal imaging the brains and eyes were excised and imaged on an 11.7T AVIII HD spectrometer (Bruker Biospin, Billerica, MA). ^{19}F quantification was performed in each rat using Voxel Tracker software (Celsense, Pittsburgh PA).

Results

Our findings revealed that two closely coupled blasts resulted in a trend of increased numbers of regions throughout the body that had ^{19}F signal, along with a concomitant increase in signal intensity, when compared to sham controls. Dietary omega-3 fatty acids (Fish Oil) have thus far shown little, if any, ability to alleviate these acute injury events. Figure 1 shows the increase in ^{19}F signal in the eyes of rats exposed to double blast compared to the sham rats and that treatment appears to have little or no effect.

Discussion

These findings demonstrate that ^{19}F MRI is effective in detecting systemic injury as a result of shock tube-generated overpressure. The injury itself is very heterogeneous, but one obvious result was that experiencing a second blast one minute after the first increased the amount of fluorine signal seen in these animals, which most likely represents increased inflammatory response (labelled macrophage accumulation), when compared with sham animals. Despite having potent anti-inflammatory properties, a diet rich in omega-3 fatty acids did not readily alleviate these acute injury events. Chronic events may be more amenable to other functions of omega-3 fatty acids, such as membrane structural restoration. More work needs to be done to explore the role that systemic inflammation has on not only acute and chronic injury, thus, we plan to extend evaluations over more prolonged post-blast intervals.

Acknowledgements

DoD grants from the MOMRP and USAMRMC/CDMRP, #: W81XWH-14-2-0178.

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1. Hanley, C. J. The Washington Post, <http://www.washingtonpost.com>. Accessed August 20, 2013.
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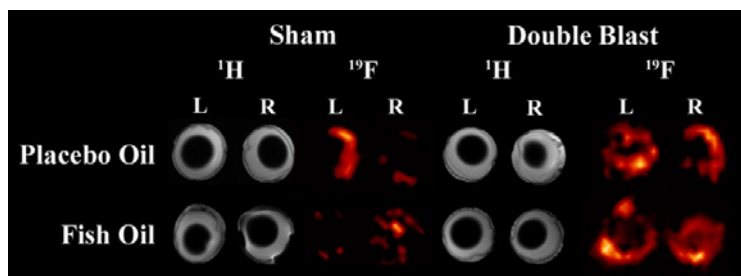


Figure 1: Representative ^1H anatomical and ^{19}F -MRI images of the eyes of rats fed omega-3 deficient (placebo) and enriched (fish oil) diets, for shams and rats exposed to double blast 3 days post-blast. ^{19}F signal is shown in a “Hot Iron” pseudo-color.

Characterization of Inflammation Induced by Exposure to Primary Blast Waves in Rats using ^{19}F MRI

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Disclaimer

- The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense
- Research was conducted under an IACUC approved protocol in an AAALACi accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 Edition.

Introduction

- Blast has been the leading cause of injury of military personnel in the conflicts in Iraq and Afghanistan (e.g. operations OEF and OIF)
- Previously, blast injury was seldom seen outside of the battlefield, but due to the increasing use of explosives by terrorists, civilian casualties are becoming more common

Types of Blast Injury

- **Primary** – blast wave and subsequent changes in air pressure
- **Secondary** – flying debris and bomb fragments, heat, and chemical gases
- **Tertiary** – body being thrown by the blast
- **Quaternary** – exacerbation or complications of existing conditions

Aim

- Our objective was to longitudinally monitor the nature and timing of immune cell guided inflammatory processes after blast, of which, principal interest was the visual system
- We also examined the impact of dietary omega-3 polyunsaturated fatty acids, known to be anti-inflammatory, on blast injury vulnerability

Methods



Adult male
Sprague Dawley rat
(30 day-old; ~150 g)

Rats are fed an omega-3 fatty acid deficient base diet for at least 4 weeks

Rats are omega-3 supplemented daily by oral gavage with 0.9 ml/kg **Fish Oil** (200 and 270 mg/kg DHA plus EPA) or Soybean Oil (**Placebo Oil**) suspended in 10% non-fat milk

Randomly Divided
into 4 groups

Placebo Oil Sham (n = 8)

Placebo Oil Double Blast (n = 11)

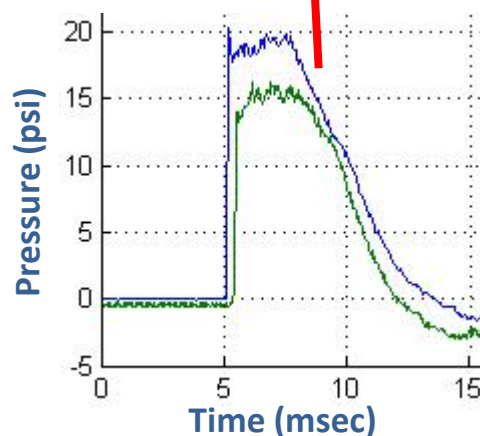
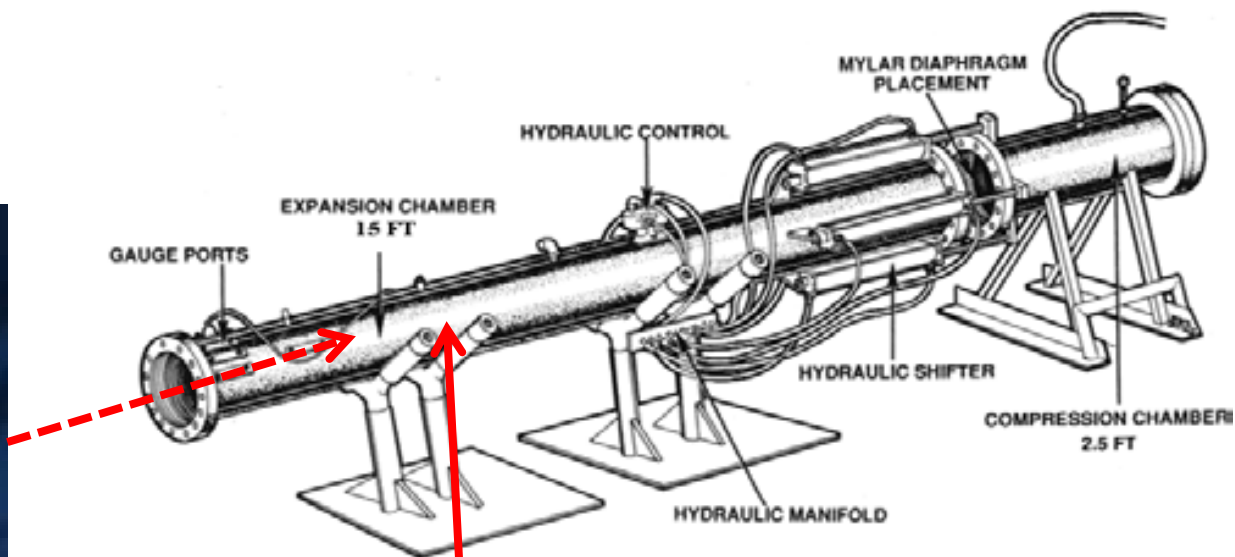
Fish Oil Sham (n = 8)

Fish Oil Double Blast (n = 12)

Compressed Air-Driven Shock Tube



Animal Holder



- Mach 1.34 shock front speed
- 62 μ sec rise time
- 281 mph (126 m/s) blast wind
- Acceleration > 1000 xg

¹⁹F MRI

- 2 days post injury animals injected with fluorine agent
 - VSense (Celsense Pittsburgh PA) 1 ml/300 g at a rate of 0.4 ml/min via tail vein
 - VSense taken up by macrophages which home to sites of inflammation and the mononuclear phagocyte system
- Animals euthanized at day 3
- Whole Animals imaged at 9.4T
- Heads only imaged at 7T
- Brains and Eyes imaged at 11.7T
- Images analysed with Voxel Tracker software
- Histology

MRI Parameters

9.4T

¹H

TE/TR = 14/4000

RARE Factor = 8

Matrix = 256 x 256

FOV = 64 x 64

Averages = 2

31 Slices

2 mm Slice Thickness

¹⁹F

TE/TR = 14/4000

RARE Factor = 8

Matrix = 64 x 64

FOV = 64 x 64

Averages = 128

31 Slices

2 mm Slice Thickness

7T

¹H

TE/TR = 28/5000

RARE Factor = 4

Matrix = 256 x 256

FOV = 35 x 35

Averages = 2

25 Slices

2 mm Slice Thickness

¹⁹F

TE/TR = 28/5000

RARE Factor = 4

Matrix = 64 x 64

FOV = 35 x 35

Averages = 512

25 Slices

2 mm Slice Thickness

11.7T

¹H

TE/TR = 15/3000

RARE Factor = 4

Matrix = 256 x 256

FOV = 25 x 25

Averages = 2

4 Slices

5 mm Slice Thickness

¹⁹F

TE/TR = 15/3000

RARE Factor = 4

Matrix = 32 x 32

FOV = 25 x 25

Averages = 2048

4 Slices

5 mm Slice Thickness

Results

It's important to confirm successful intravenous delivery of contrast agent ^1H anatomical images of the tail and toes of rear feet overlaid with the ^{19}F images rendered in 'HotIron' pseudocolor

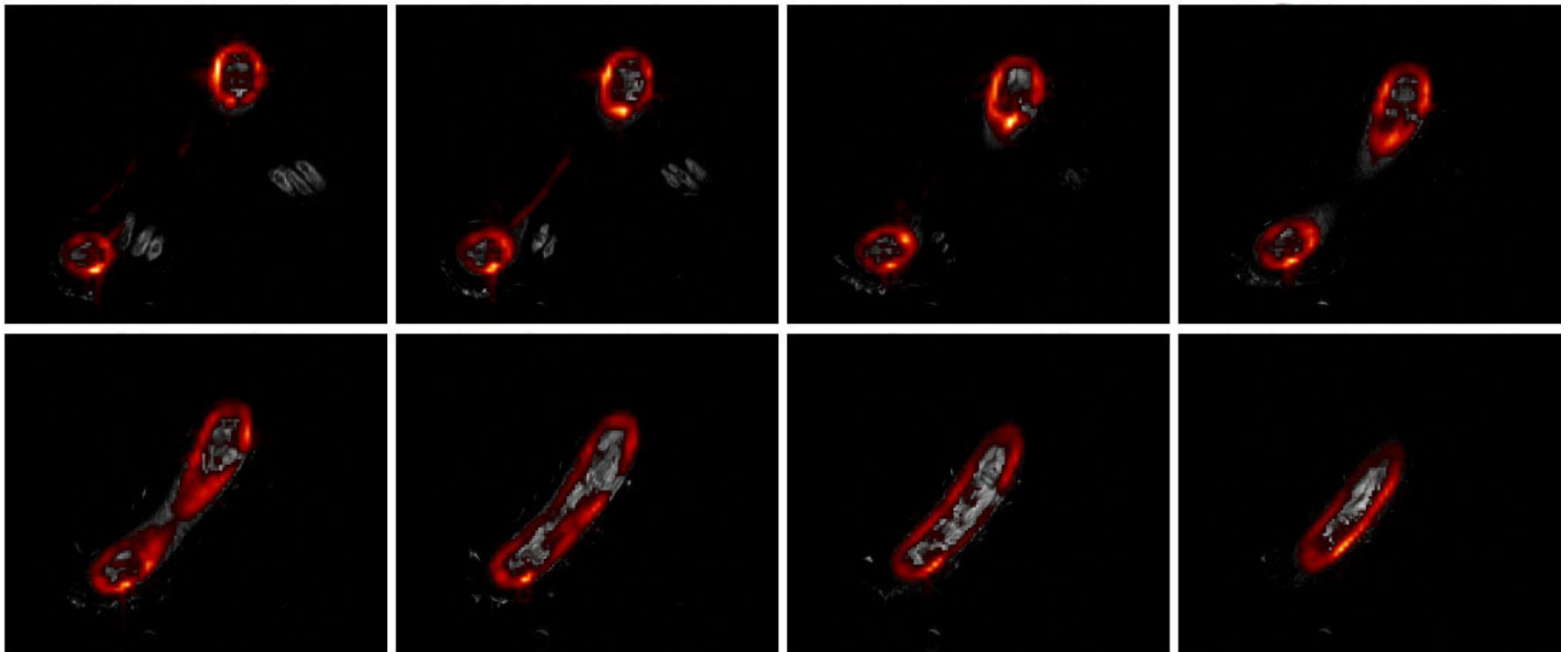
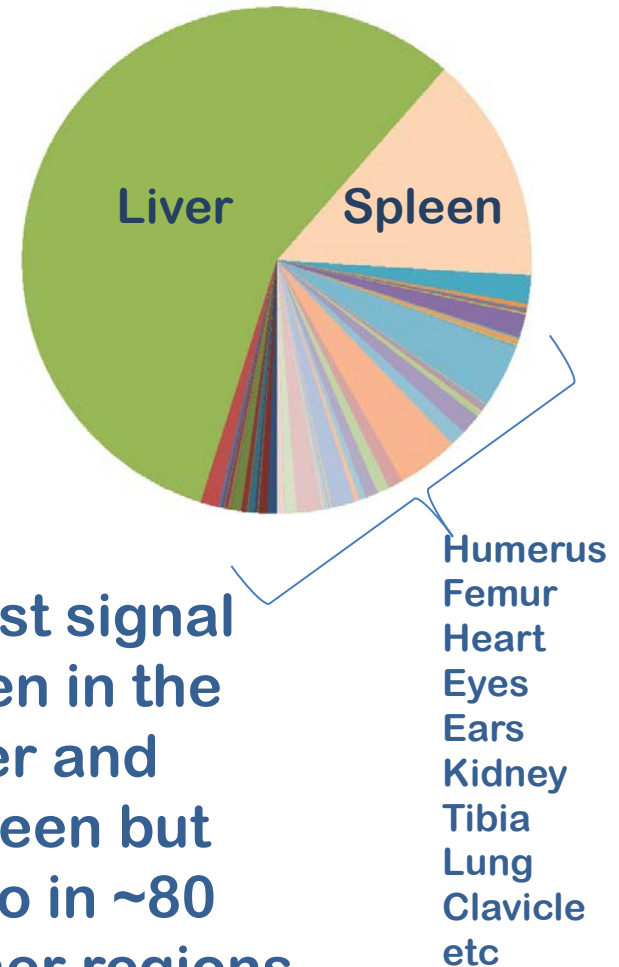


Image easily identifies failed tail vein injection with contrast agent sub-dermally in the tail

Results

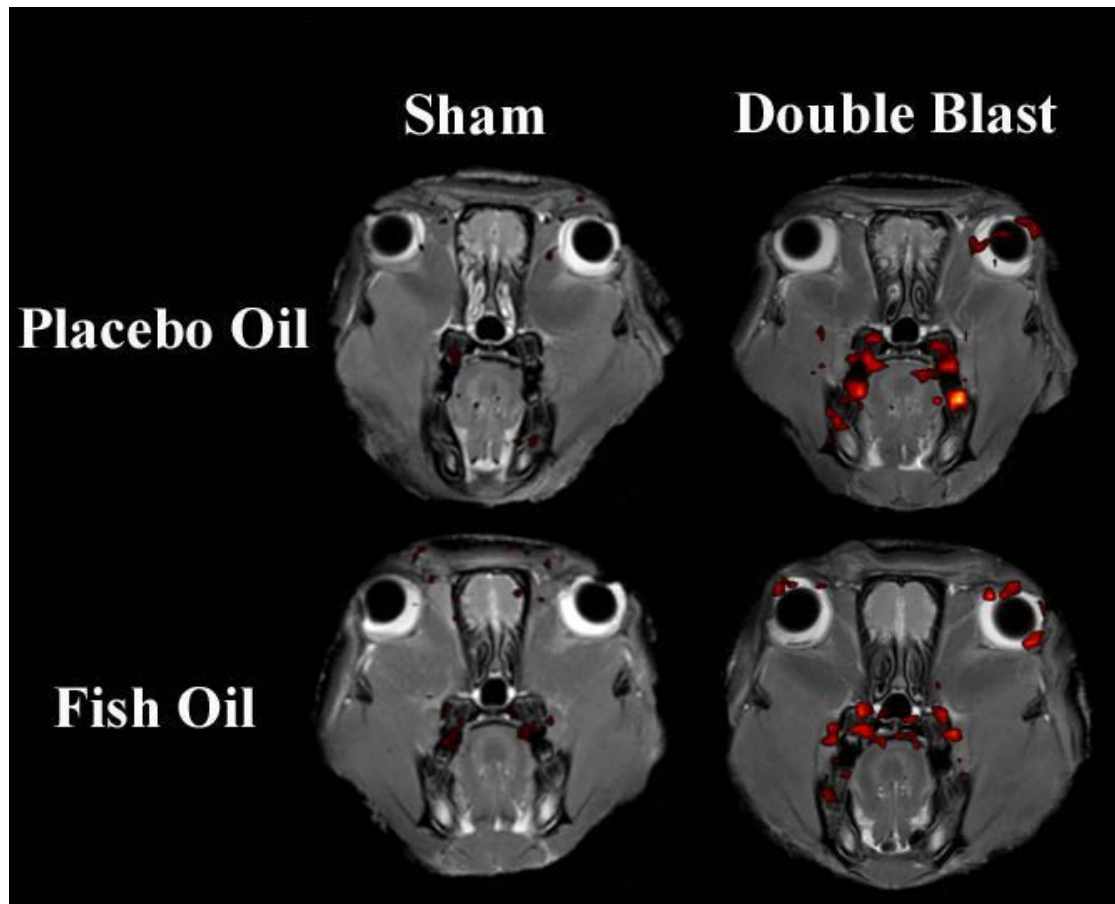


^{19}F signal is observed in numerous anatomies throughout the body



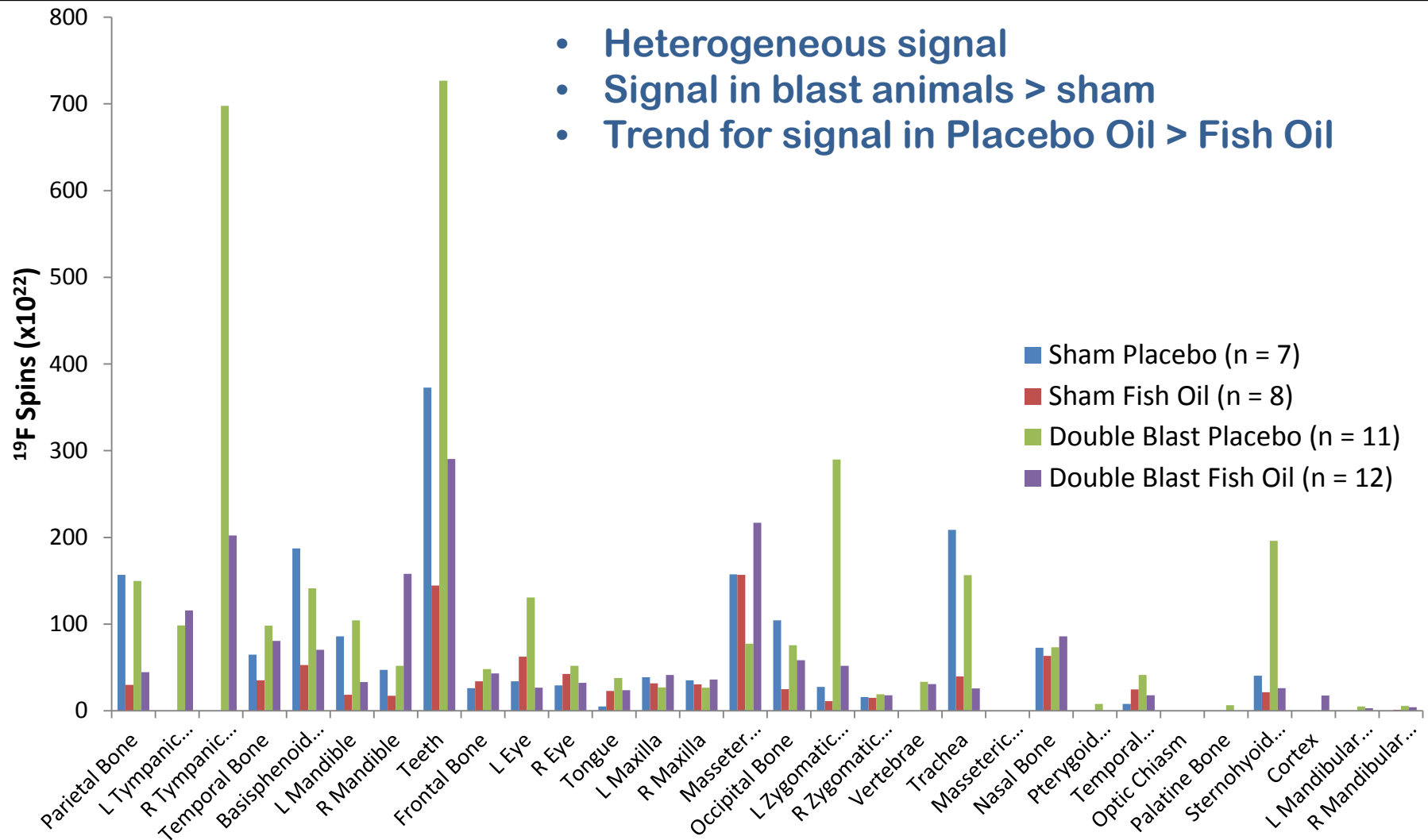
Most signal seen in the liver and spleen but also in ~80 other regions

Head Images

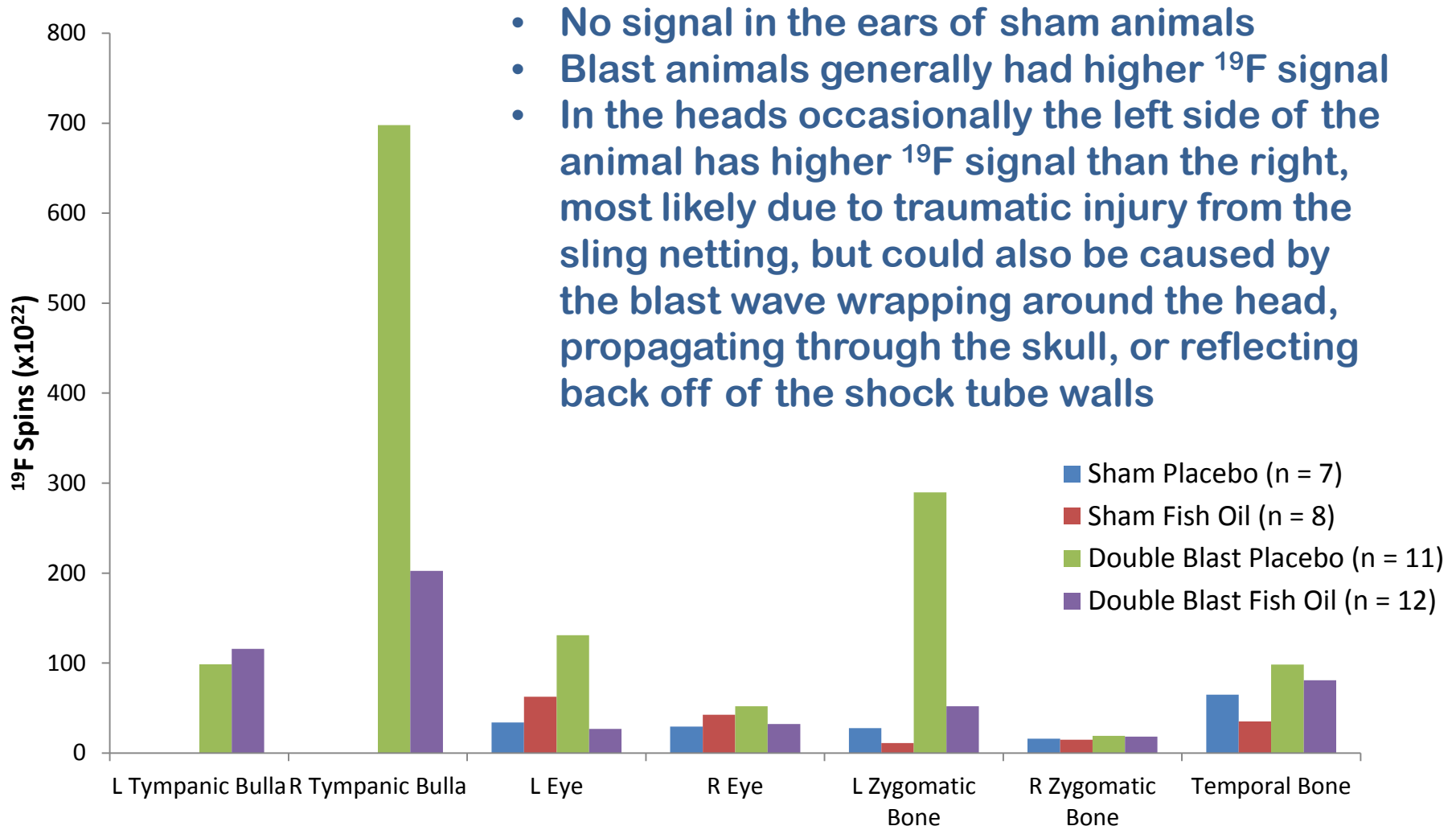


Representative images show that the Double Blast rats have more ^{19}F signal in the head within the areas of the eyes and the teeth

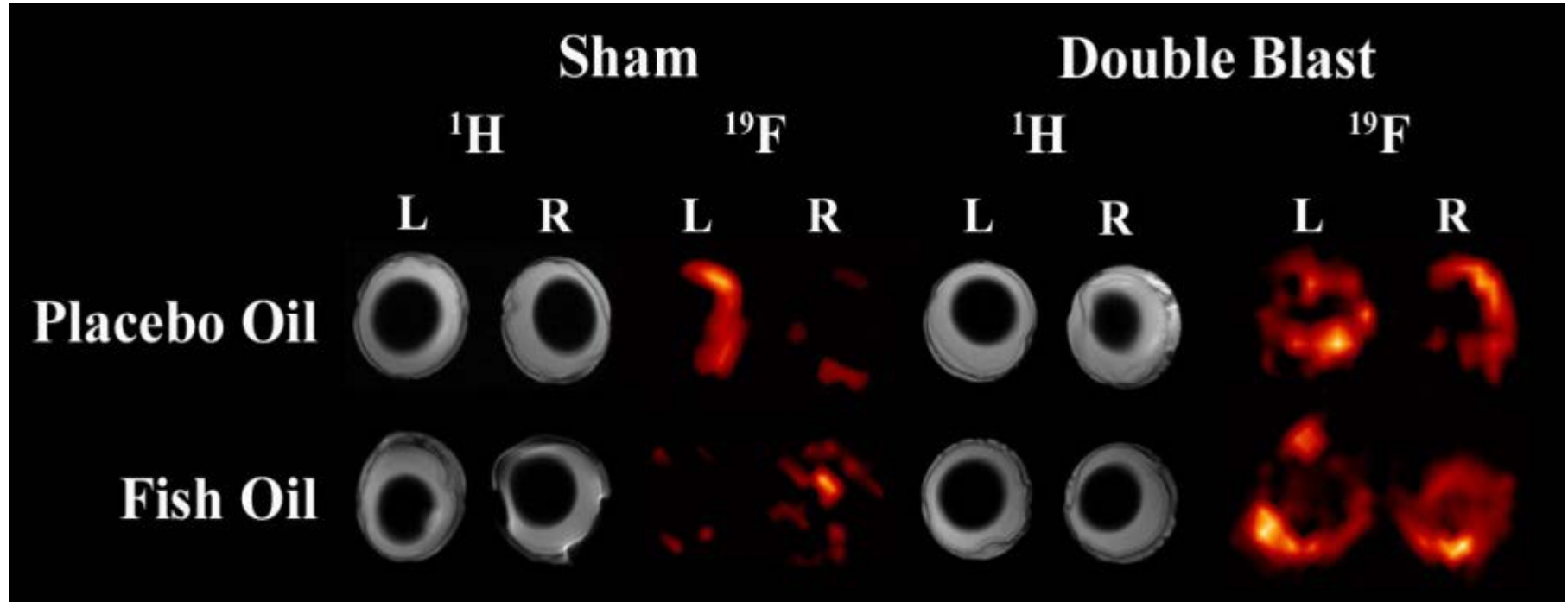
Head Analysis



Focused Head Examples

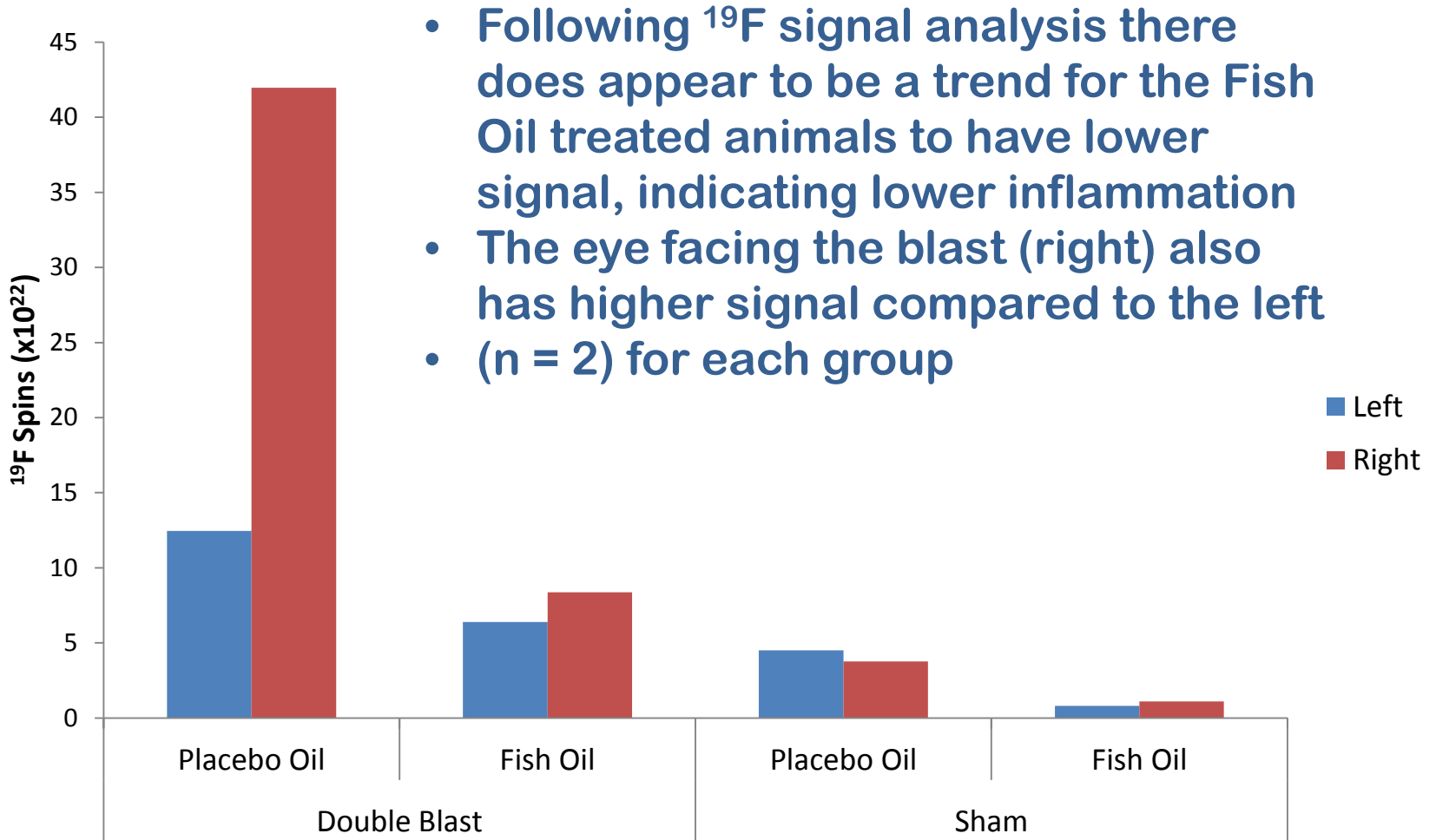


Eye Images



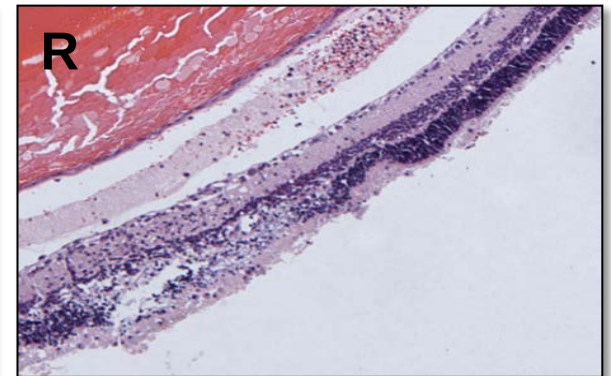
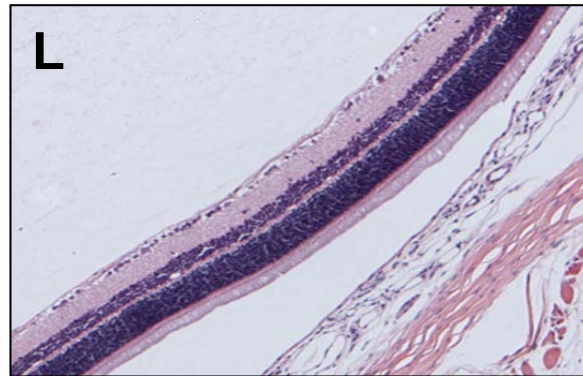
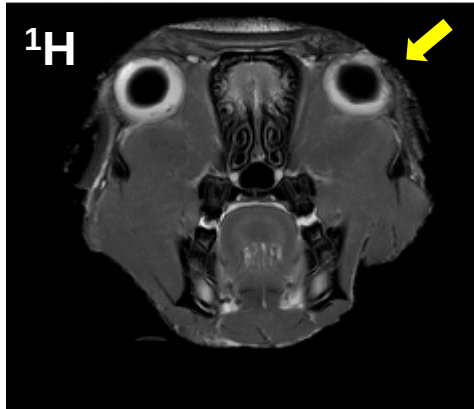
Double blast rats have increased ^{19}F signal in the eyes, compared with shams. There appears to be no difference between treatment groups, or the eye facing toward the blast (right) or away from it (left)

Eye Analysis

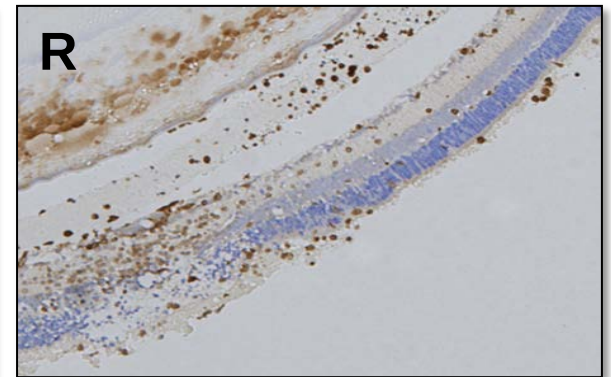
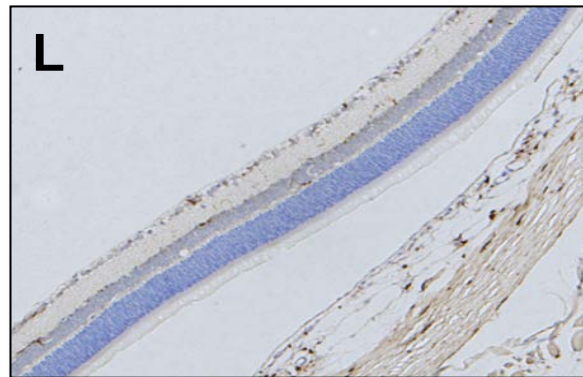
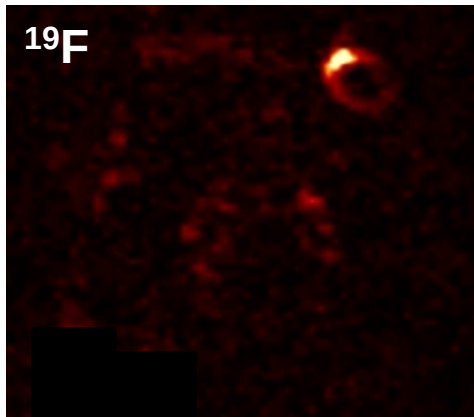


Retina Histopathology

Retina; H&E Stain



Retina; Iba-1 IHC



Retina histopathology for a placebo oil treated rat for H&E stain for degenerating neurons (e.g. photoreceptors) and Iba-1 IHC for microglia and macrophages is shown.

Conclusions

- Current findings with ^{19}F -MRI macrophage tracking and histopathology in rats reveal that immune cells involved in neurodegeneration and neuroinflammation processes, i.e., macrophages and microglia, accumulate in various body regions following exposure to blast waves
- Dietary omega-3 fatty acids, as given by high dose fish oil, have so far shown a modest ability to alleviate these injury events, despite having potent anti-inflammatory properties targeting immune cell activities
- Reason for lack of a robust treatment effect is unclear, but may be reflective of the injury time frame examined i.e., acute phase. Thus, we plan to extend evaluations over more prolonged post-blast intervals (eg 28 days post)

Acknowledgements

- DoD grants from the MOMRP and USAMRMC/CDMRP, #: W81XWH-14-2-0178

Cytokine Responses in a Rat Model of Blast-induced Mild Traumatic Brain Injury (mTBI)

Chemokines and cytokines play early pivotal roles in the inflammatory cascades underlying blast-induced injuries and are promising targets for therapeutic interventions. To effectively pursue this therapeutic avenue, the timing of the interplay among these responses must be characterized to identify the key participants and the optimal therapeutic windows for intervention. Researchers at the Walter Reed Army Institute of Research (WRAIR; Silver Spring, Maryland) are conducting a study that includes longitudinal screening of cytokine levels in plasma and brain at varied times after blast exposure using immunoassay arrays based on newly developed Luminex® bead technology. The arrays (R&D Systems Inc.) are used to simultaneously and precisely quantify very small concentrations (picomolar) of up to 17 rat specific cytokines across a single 96 sample well plate. Thus, this method is highly time- and cost-effective versus data yield. Analyses to date of plasma and brains collected from blast-exposed rats reveal significant increases (2-fold or less) in pro-inflammatory cytokines (Chemokine (C-X-C motif) ligand 2 (CXCL2), interleukin (IL) -1- α , IL-18, and tumor necrosis factor (TNF) - α) along with counter elevations in inflammation resolving cytokines (IL-4 and tissue inhibitor of metalloproteinases (TIMP) -1) up to seven days post-exposure (Figure X-1; refs. 1, 2). Magnetic resonance imaging (MRI) has shown that extensive immune cell infiltration occurs within brain and retina by three days post-exposure (refs. 3, 4, 5). These findings have been corroborated by immunohistochemistry of brain and eye sections using biomarkers for activated immune cells (refs. 3, 4, 5). Cytokines can act as recruitment factors for macrophages into tissues, and in turn are excreted by these immune cells as signaling molecules that further trigger protein-pathways involved in apoptosis of neurons (e.g., caspases). Based upon these response profiles, interventions with existing compounds targeting these mediators are likely to be most effective during subacute or acute phases of injury. Notably, since rats maintained on a diet supplemented with long chain omega-3 fatty acids (i.e., fish oil) appear to have lowered (23 percent or less) pro-inflammatory cytokines (CXCL3, Intercellular Adhesion Molecule (ICAM) -1, IL-1- α , IL-6, IL-18, and TNF- α) in the plasma and brain (Figure X-1; refs. 1, 2), our data points to the potential utility of these diets as nutritional anti-inflammatory countermeasures to blast-induced TBI.

By defining important neurobiological underpinnings of blast injuries, these findings point to potential therapeutic countermeasures that can lessen permanent debilitations suffered by Service members experiencing blast-induced injuries.

This research was funded by the Vision Research Program (VRP) managed by the Congressional Directed Medical Research Program (CDMRP) (award W81XWH-14-2-0178).

Figure X-1

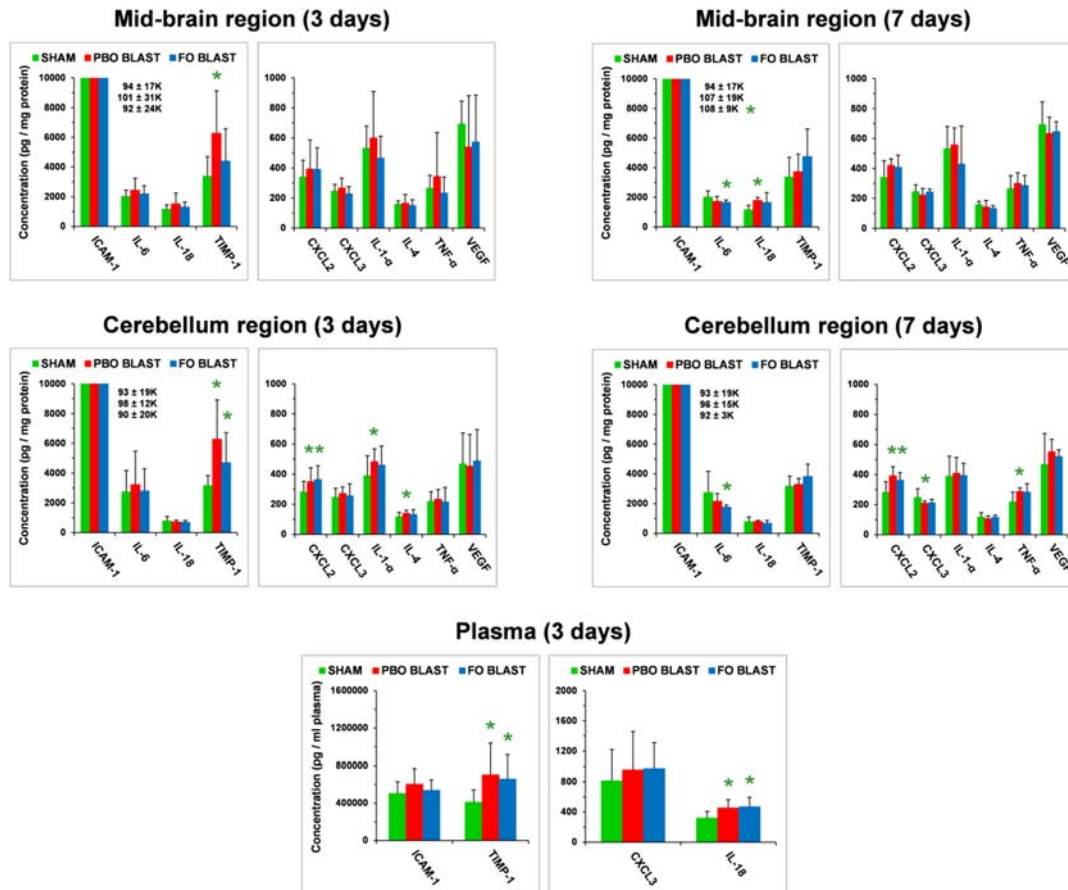


Figure legend: Bar graphs for the mid-brain (top 2 panels), cerebellum (middle 2 panels), and plasma (bottom panel) cytokine levels of placebo versus fish oil treated rats, at 3 and 7 days following double blast exposure, as well as those for sham controls (shams = green, PBO; placebo = red, and FO; fish oil = blue; n = 15, 11, and 10 and 15, 4, and 4, as by day, respectively). For each tissue, the data is broken into two rescaled frames to allow visualization of less abundant cytokines. Tissue concentrations (per mg total protein or ml plasma) for up to 10 cytokines are shown, i.e., CXCL2, CXCL3, ICAM-1, IL-1- α , IL-4, IL-6, IL-18, TIMP-1, TNF- α , and VEGF. There were no significant differences detected between dietary treatment groups for blasted animals. *p < 0.05; significant difference between shams and blasted rats, as by t-test. (Figure used with permission from the authors)

Reference:

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