Docosahexaenoic acid (DHA) Reduces Traumatic Axonal Injury in a Rodent Head Injury Model

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ABSTRACT

Traumatic brain injury (TBI) remains the most common cause of death in persons under age 45 in the Western world. Recent evidence from animal studies suggests that supplementation with omega-3 fatty acids (O3FA) improves functional outcomes following focal neural injury. The purpose of this study is to determine the benefits of DHA supplementation following diffuse axonal injury in rats. Four groups of ten (n=40) adult male Sprague-Dawley rats were subjected to an impact acceleration injury and then received 30 days supplementation with either 10mg/kg/day or 40mg/kg/day of docosahexaenoic acid (DHA). Serum fatty acid levels were determined from the isolated plasma phospholipids prior to the injury and at the end of the 30 days of DHA supplementation. Following sacrifice, brainstem white matter tracts underwent fluorescent immunohistochemical processing for labeling of beta amyloid precursor protein, a marker of axonal injury. Dietary supplementation with either 10mg/kg/day or 40mg/kg/day of DHA for 30 days results in significantly ($p<0.05$) increased DHA serum levels of 123 and 175% over baseline, respectively. Immunohistochemical analysis reveals significantly ($p< 0.05$) decreased numbers of amyloid precursor protein positive axons in animals receiving dietary supplementation with DHA, 26.1 (S.D. 5.3) and 19.6 (S.D.4.7) axons per mm^2 respectively; versus 147.7 (S.D. 7.1) axons in unsupplemented animals. Sham injured animals had 6.4 (S.D. 13.9) APP positive axons per mm^2. Dietary supplementation with DHA increases serum levels in a dose response effect. DHA supplementation significantly reduces the number of APP positive axons at 30 days

postinjury to levels similar to uninjured animals. DHA is safe, affordable, and readily available worldwide to potentially reduce the burden of traumatic brain injury.

INTRODUCTION

Traumatic brain injury (TBI) is recognized as one of the most common causes of death in persons under age 45, with a large societal impact in terms of resource utilization and health care costs, while the human suffering is incalculable. Each year in the United States, there are over 2 million cases, 220,000 hospitalizations, and 52,000 deaths from head trauma; while another 80-90,000 persons each year suffer permanent debilitation. The total cost of TBI in the United States, both direct healthcare and indirect personal and societal costs, is estimated at $44 billion per annum (Waxweiler RJ et al., 1995, Sosin et al., 1995). While the causal mechanisms of TBI cross a spectrum from low height falls to sports related head impacts to blunt high speed motor vehicle injuries, the underlying pathophysiology remains similar. Autopsy studies from patients with injury classifications ranging from concussion to severe traumatic brain injury often demonstrate diffuse injury to white matter tracts running from the cortex to the brainstem. The extent of traumatic axonal injury (TAI) is a principal determinant of morbidity and mortality following traumatic brain injury.

While various studies have given hope that pharmacological or physiological interventions may reduce axonal injury, to date over 30 major clinical studies have failed to show a significant effect in reducing the morbidity and mortality or improving

outcomes of TBI (Doppenberg EM et al., 1997). Advances in treatment strategies in the clinical management of the acute injury, critical care and rehabilitation medicine starting have greatly improved both the survival and functional outcomes. The science of nutritional supplementation has undergone significant change from early goals of simply delivering necessary calories to current regimens which provide specific amino acid and fatty acid combinations to maximize the healing process. Docosahexaenoic acid (DHA), the principle constituent O3FA of neural tissue, demonstrates significant anti-inflammatory properties and is the precursor of Neuroprotectin 1. Recent evidence from animal studies suggests that supplementation with O3FA (particularly eicosapentaenoic acid (EPA) and DHA) improves functional outcomes following focal neural injury (Wu, A et al., 2004, 2009, Mills et al, submitted for publication). Additional recent evidence from animal models of ischemia demonstrate improvement in outcome and reduction in infarct volume with DHA supplementation (Belayev, 2009). We hypothesized that DHA supplementation following diffuse axonal injury in rats would ameliorate secondary mechanisms of injury and result in a decreased number of injured axons, as measured using immunohistochemical analysis of amyloid precursor protein positive axons.

METHODS

Marmarou impact acceleration injury model in rats

Four groups of ten (n=40) of adult male Sprague-Dawley rats were subjected to a Marmarou impact acceleration injury resulting in reproducible traumatic brain injury. Rats weighing between 350 and 400 grams received isoflurane induction anesthesia and subsequently maintained on inhaled isoflurane using a modified medical anesthesia machine. Lidocaine 1% local anesthetic was injected subcutaneously along the planned incision site. Buprenorphine was used for postoperative analgesia. Body temperature was controlled during the approximately 10 minute procedure using a heating blanket, and adequate sedation was confirmed by evaluation of response to heel tendon pinch. The animals were shaved and prepared in sterile fashion for surgery, followed by subcutaneous injection of local anesthetic into the planned incision site. A 3cm midline incision in the scalp was made, periosteal membranes separated, exposing bregma and lambda. A metal disk 10mm in diameter and 3mm thick was attached to the skull with cyanoacrylate and centered between bregma and lambda. The animal is placed prone on a foam bed with the metal disk directly under a plexiglas tube. A 450-gram brass weight is dropped a single time through the tube from a height of 2 meters striking the disk. The animal is then ventilated on 100% O_2 while the skull is inspected, the disk removed, and the incision repaired. When the animal recovers spontaneous respirations, anesthesia is discontinued and the animal is returned to its cage for postoperative observation (Marmarou et al., 1994). All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of West Virginia University, and are performed according to the principles of the Guide for the Care and Use of Laboratory

Animals, published by the Institute of Laboratory Resources, National Research Council (NIH publication 85-23-2985).

DHA supplementation and serum level monitoring

The four groups received dietary supplementation with DHA (Martek Inc, Columbia, Maryland) for 30 days starting on postinjury day 1. Two of the four groups received dietary supplementation DHA daily in the following amounts: Group 1, 10 mg/kg/day; Group 2, 40 mg/kg/day. These dosages were selected based on our previous studies of O3FA supplementation, and reflect typical human equivalent dosing. Groups 3 served as an unsupplemented control, Group 4 underwent sham injury and received no supplementation. Each group received rat chow ad lib and were housed in the small animal vivarium under veterinary staff supervision. Fatty acid blood testing was performed prior to the injury and at the end of the 30 days of DHA supplementation by analyzing the isolated serum phospholipids (including DHA, EPA and arachidonic acid) from 50 microliter blood samples using a previously described method (Holub and Skeaff, 1987) (Nutrasource Diagnostics, University of Guelph, Ontario, Canada).

Tissue Preparation and Immunhistochemical Labeling

Following 30 day post-injury survival animals were euthanized with a lethal dose injection of 0.5ml Ketamine and 0.5ml Xylazine. The animals were immediately perfused transcardially with 200ml cold 0.9% saline to wash out all blood. This was followed by 4% paraformaldehyde infusion in Millonigs buffer for 40 minutes. The entire brain, brainstem, and rostral spinal cord were removed and immediately placed in 4%

paraformaldehyde for 24 hours. Following 24 hours fixation, the brain was blocked by cutting the brainstem above the pons, cutting the cerebellar peduncles, and then making sagittal cuts lateral to the pyramids. The resulting tissue containing the corticospinal tracts and the medial lemnisci, areas shown previously to yield traumatically injured axons, was then sagitally cut on a vibratome into 50 micron thick sections. The tissue underwent temperature controlled microwave antigen retrieval using previously described techniques (Stone JR et al., 1999). The tissue was preincubated in a solution containing 10% normal serum and 0.2% Triton X in PBS for 40 minutes.

For amyloid precursor protein labeling, the tissue was incubated in polyclonal antibody raised in rabbit against beta amyloid precursor protein (APP) (#51-2700, Zymed) at a dilution of 1:200 in 1% NGS in PBS overnight. Following incubation in primary antibody, the tissue was washed 3 times in 1% NGS in PBS, then incubated in a secondary anti-rabbit IgG antibody conjugated with Alexa 488 fluorophore (A11008, Molecular Probes), diluted at 1:200 for two hours. The tissue underwent a final wash in 0.1M phosphate buffer and then was mounted using an antifade agent and coverslipped. The slides were sealed with acrylic and stored in the dark in a laboratory refrigerator (Mills et al., 2003)).

Fluorescent Microscopy and Image analysis

The tissue was examined and images acquired using a laser scanning confocal microscope system (Zeiss) with an Argon 488 excitation laser and a 40x objective lens. Ten digital images are obtained from the tissue of each animal and images were then

randomized. Individual injured axons were independently counted and data was stored in a spreadsheet (Microsoft Corp.). Counts were converted to density per mm2 by the formula axon count per image / image area. Differences between group means were determined using paired t-tests and considered significant if the probability value was less than 0.05.

Stereological Quantification of axonal injury

A stereological method was used to determine an unbiased estimate of the number of APP positive axons per cubic mm in the corticospinal tract and medial lemniscus. The optical fractionator technique utilitizing StereoInvestigator 7.0 (MBF Bioscience) and a Nikon Eclipse microscope with 4x and 20x objectives was performed. Sagittal APP stained specimens were examined with low magnification and regions of interest were drawn incorporating the corticospinal tract and medial lemniscus. The software then selected random 50μm^2 counting frames with depth of 15μm, and APP positive axons were marked. The volume of the ROI was determined using the Cavalieri method, the volume of the sum of the counting frames was calculated, the sum total of injured axons within the counting frames was calculated, and an estimate of the number of APP positive axons per cubic mm was calculated.

RESULTS

Impact Acceleration Model and Serum Fatty Acid Levels

The mortality rate in this model of traumatic axonal injury was 0%. Animals tolerated daily oral supplementation without any observed untoward effects.

Supplementation for 30 days after the brain trauma with DHA at dosage of either 10mg/kg/day or 40mg/kg/day resulted in increased levels of serum DHA of 123 and 176% over initial levels, respectively (Figure 1.). Animals receiving no supplementation had a 7% decrease in DHA. Serum EPA levels likewise increased in supplemented animals 104 and 313%, respectively; while unsupplemented animals showed a 59% decrease. The AA:EPA ratio, a marker of inflammation, decreased 72 and 109%, respectively, while increasing 65% in unsupplemented animals.

Immunohistochemical analysis of APP positive axons

In sham injured animals, axons throughout the medullary corticospinal tract and medial lemnsci demonstrated a paucity of labeling for APP. These rare labeled axons did not demonstrate vacuolization, swelling, or breakdown; typical characteristics of traumatic axonal injury (Figure 2). In comparison, evaluation of axons from animals which received no supplementation 30 days postinjury demonstrated focal labeling of APP within swollen contiguous and terminal axon segments, consistent with previous findings suggestive of impaired axoplasmic transport in traumatic axonal injury. Following microscopic digital image acquisition from multiple areas within the corticospinal tract and medial lemnsci from multiple tissue slices, counting of APP positive axons was

performed, and results were converted to density per mm^2. This demonstrated a significant quantitative difference of 147.7 (S.D. 7.1) axons in unsupplemented animals versus sham injured animals which had 6.4 (S.D. 13.9) APP positive axons per mm^2 (Figure 3).

In animals receiving either 10mg/kg/day or 40mg/kg/day of DHA, axons throughout the corticospinal tract and medial lemnisci demonstrated only rare APP positive axons, similar to sham injured animals. However, in comparison to sham injury animals, the rare APP positive axons were more likely to demonstrate morphologic characteristics of injury, primarily swelling and disconnection. Quantitative analysis reveals significantly ($p< 0.05$) decreased numbers of APP positive axons in animals receiving dietary supplementation with DHA, 26.1 (S.D. 5.3) and 19.6 (S.D. 4.7) axons per mm^2 respectively; versus 147.7 (S.D. 7.1) axons in unsupplemented animals.

Immunohistochemical analysis of Caspase 3 positive axons

Following microscopic digital image acquisition from multiple areas within the corticospinal tract and medial lemnsci from multiple tissue slices, counting of caspase 3 positive axons was performed, and results were converted to density per mm^2. This demonstrated a significant quantitative difference of 124.9 (S.D. 37.0) axons in unsupplemented animals versus sham injured animals which had 1.7 (S.D 7.5) caspase 3 positive axons per mm^2 (Figure 4). In animals receiving either 10mg/kg/day or 40mg/kg/day of DHA, axons throughout the corticospinal tract and medial lemnisci demonstrated only rare caspase 3 positive axons, similar to sham injured animals.

Quantitative analysis reveals significantly ($p < 0.05$) decreased numbers of caspase 3 positive axons in animals receiving dietary supplementation with DHA, 2.8 (S.D. 1.7) and 0.9 (S.D. 3.7) axons per mm^2 respectively; versus 124.9 (S.D. 37.0) axons in unsupplemented animals.

Stereologic Analysis of APP Positive Axons

The stereologic optical fractionator method was utilized to determine an unbiased estimate of the number of APP positive axons per cubic mm within the corticospinal tract and medial lemnsicus. This demonstrated a significant quantitative difference of 128,048 (S.D.42,206) axons in unsupplemented animals versus sham injured animals which had 1648 (S.D. 1847) APP positive axons per mm^3 (Figure 4). In animals receiving either 10mg/kg/day or 40mg/kg/day of DHA, stereologic analysis reveals significantly ($p < 0.05$) decreased numbers of APP positive axons in animals receiving dietary supplementation with DHA, 3720 (S.D. 1924) and 2892 (S.D. 3221) axons per mm^3 respectively; versus 128,048 (S.D. 42,206) axons in unsupplemented animals.

DISCUSSION

This study demonstrates that DHA alone**,** given after traumatic axonal injury**,** is neuroprotective. Oral supplementation with either 10mg/kg/day or 40mg/kg/day of algae-derived DHA for 30 days following an impact acceleration injury resulted in significantly decreased numbers of injured axons as measured by amyloid precursor protein staining. Likewise, a significant decrease in active caspase-3 positive axons provides additional evidence for the neuroprotective and injury ameliorating effects of DHA.

Serum response

Analysis of the serum phospholipids at the end of the supplementation period after the traumatic insult showed a dose-response effect by an increase in the total of both EPA and DHA levels as compared to the sham animals, whereas there was a decrease in the combined EPA and DHA serum levels in the non-supplemented animals. These results are consistent with previous studies in both rats and humans which demonstrate uptake and retroconversion of DHA into EPA (Vidgren et al, 1997, Brossard et al, 1996). Interestingly, there was an increase in the serum arachidonic acid levels in the non-supplementated group although non- significant increase was seen in the supplemented animals. As a result, the AA/EPA ratio, an indicator of systemic inflammation, significantly increased in the non-supplemented animals compared to the supplemented animals. This would have the effect of activating leukocytes into neutrophils and macrophages that could more easily enter into the brain. Dosage with 10mg/kg/day or 40mg/kg/day reflect typical human equivalent DHA supplemental dosing with between 1 and 3 grams per day.

Mechanisms of TBI

Conventional theory has held that TAI involves immediate axonal tearing through the direct action of forces associated with the traumatic insult. More recently, experiments employing anterograde tracers have revealed that traumatic axonal injury is a progressive event involving a focal impairment of axoplasmic transport leading to axonal swelling and ultimately disconnection following TBI (Raghupathi R et al., 2000). The cellular mechanisms of injury include mitochondrial disruption, loss of calcium homeostasis, and activation of apoptotic cascades (Wang HG et al., 1999, Buki A et al., 2000, Eldadah et al., 2000).

The concept of the secondary phase of TBI includes what are now recognized as ongoing abnormalities in glucose utilization, cellular metabolism, as well as membrane fluidity, synaptic function, and structural integrity (Hovda, 2007, Aoyama et al, 2008). In general, axon membranes are injured, leakage occurs and axonal transport is interrupted in a progressive process. This concept is reinforced by recent autopsy findings in professional contact sports athletes showing multi-focal areas of damaged neurons and their processes, remarkable for tau antibody staining, representing numerous times and regions of injury from multiple concussions (Omalu et al, 2005, 2006).

Omega-3 Fatty Acids

The primary O3FA's are EPA and DHA. Prior work from our lab with the same head injury model has shown that dietary supplementation with a fish oil concentrate rich in

EPA and DHA significantly reduces the number of injured axons (Mills et al, submitted for publication). This current experiment is the first to analyze the effects of immediate post-injury treatment with an algal derived DHA- only dietary additive. DHA constitutes the primary O3FA fatty acid in brain synaptosomal plasma membranes and synaptic vesicles. Human brain O3FA content consists of 97% DHA, while the retina is 93% (Martinez, 1992). Neuronal membranes are composed largely of phospholipids, and DHA stimulates phosphatidylserine and phosphatidylethanolamine concentrations as well as neurite outgrowth triggered by nerve growth factor (Cao et al 2004). During fetal development and the first few years of life, the DHA content of the human brain exponentially rises, while dietary deficiency of DHA causes the brain to retain DHA longer when other organs are depleted. Unlike arachidonic acid (AA), DHA appears to be retained by neuronal membrane phospholipids, as opposed to astroglial cells which readily release it, probably reflecting a trophic or anti-apototic effect important for neuronal survival (Salem et al, 2001, Carver 2001, Martinez 1994). TBI can cause acute and long-lasting disturbances in brain phospholipid metabolism, and degradation of neuronal and astrocytic membrane phospholipids is documented (Wu et al, 2004) thus contributing to the evolving secondary injury.

Neuronal and cellular response to TBI

Modulation of the inflammatory cascade by O3FA has been proposed as a critical neuroprotective mechanism. Arachidonic acid, a primary omega-6 fatty acid in the plasmalemma, is metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to pro-inflammatory eicosanoids, such as two-series prostaglandins and

thromboxanes, prostaglandin E_2; and leukotriene B_4. These eicosanoids enhance vascular permeability, increase local blood flow, increase infiltration of leukocytes, and enhance production of proinflammatory cytokines such as tumor necrosis factor α, interleukin 1 (IL-1) and IL-6. In contrast, O3FA can decrease COX activity and inhibit the formation of proinflammatory eicosanoids and cytokines (Calder, 2003; Lonergan et al., 2004).

Several mechanisms have been proposed to explain how O3FA's may play a neuroprotective role including reduction in excitotoxicity, modulation of calcium and potassium channels, activation of gene transcription, formation of neuroprotectin-1 and resolvins. In various ischemia models, it has been demonstrated that DHA shows a neuroprotective effect on hippocampal injury by reduction of lipid oxidative damage through inhibition of prostaglandin synthesis, a protective mechanism against glutamate-induced neurotoxicity (Wang et al, 2003, Hogyes et al., 2003), and a reduction of reactive oxygen species seen in traumatic brain injury reduce levels of brain-derived growth factor (BDGF), synapsin I and cAMP responsive element-binding protein (CREB), which unchecked can result in synaptic dysfunction and cognitive impairment (44). The neuroprotective effect of DHA may involve activation of gene transcription through retinoid receptor signaling (Jump, 2002), and to the formation of mediators such as 10,17S-docosatriene (neuroprotectin D1), an endogenous compound with anti-oxidant (Bazan, 2005) as well as anti-inflammatory (Mukherjee et al., 2004) effects.

O3FA have significant affinity for two-pore potassium channels, such as TWIK-related potassium channel (TREK) and TWIK-related arachidonic acid-stimulated potassium

channel (TRAAK) (Lauritzen et al., 2000; Emsley et al., 2003). Both EPA and DHA have also been demonstrated to inhibit calcium channels (Danthi et al, 2005) This could potentially avert apoptosis of damaged neurons and their projecting fibers. EPA increases the levels of resolvins, thus further decreasing the intensity of the inflammatory process (Schwab et al, 2007) A recent finding shows reduction in the levels of silent information 2 (Sir2) and energy metabolic markers following O3FA supplementation. Sir2 is believed to have neuroprotective abilities in a traumatically stressed environment by reducing oxidative stress in the hippocampus. Sir2 can detoxify reactive oxygen species and modulate brain energy metabolism to ensure optimal neuronal survival (Wu, 2007). In hippocampal neurons, DHA has been shown to have neurite growth-promoting effects (Calderon and Kim, 2004).

Nutritional supplementation

Emerging modern research indicates a potentially greater role for dietary supplementation for treating patients with MTBI. Several mechanisms, as highlighted above, have been proposed for the mechanism of action of O3FA's, all potentially interacting at the cellular level and reducing the effects of traumatic insults on neurons and their projections. The findings of this study, that DHA alone may have equally dramatic results as compared to our prior work utilizing fish oil concentrate containing both EPA and DHA, have several implications. Among the most important are that DHA alone may have sufficient properties to provide significant amelioration of the TBI effects, including the additional finding that retroconversion from DHA to EPA occurs probably leading to beneficial effects of the former. This may provide several advantages including that DHA may be obtained from an algal source, it can be produced in limitless supply in a controlled laboratory environment, and be known to be free of possible contamination. Compared to marine sources, there is elimination of many issues including procurement and purity.

The numerous proposed mechanisms of action of O3FA demonstrate that the biochemical pathways may either be unknown or multiple in their effects of reducing traumatic axonal damage. However, the most likely manner in which these positive effects are mediated appears to involve stabilization of the cellular environment, to reduce reactive oxygen species, to modulate continued energy production, and to improve upon axonal injury and repair mechanisms. Our findings are consistent with previous research which has demonstrated that O3FA are protective against cellular injury (Wu et al, 2007).

O3FA preparations have been shown previously to be safe and well tolerated in patients in several disease states (Schlanger et al., 2002). Further research is needed to ascertain the exact mechanisms, dosages, and preferred administration schedule for prevention of MTBI, including a pre-treatment protocol which is currently underway in our laboratory. DHA concentrates, as in this experiment, derived from algal sources are commercially available for clinical use in TBI. Administration of DHA after brain trauma as a neuroprotective and injury ameliorating treatment deserves consideration and further clinical investigation as a promising, inexpensive, and innovative approach in TBI management.

CONCLUSION

Dietary supplementation with DHA increases serum levels of these same fatty acids. DHA supplementation significantly reduces the number of APP positive axons at 30 days postinjury to levels similar to uninjured animals. DHA is safe, affordable, and readily available worldwide to potentially reduce the burden of traumatic brain injury.

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FIGURES:

Figure 1. Oral Supplementation with either 10mg/kg/day or 40mg/kg/day of DHA for 30 days increased serum levels of DHA and total O3FA. The AA/EPA ratio, a marker of systemic inflammation, was significantly lower in animals receiving supplementation as compared to unsupplemented animals.

Figure 2. (a) Fluorescent immunohistochemical images of brainstem sagittal sections labeled with APP antibody showing multiple swollen, disconnected axons in corticospinal tracts and medial lemnisci in animals subjected to impact acceleration injury 30 days prior. (b) Sham injured animals demonstrated only rare APP positive axons. Animals receiving either 10mg/kg/day (c) or 40mg/kg/day (d) of DHA for 30 days postinjury demonstrated a paucity of injured axons, similar to sham injured animals. Bar = 5 μm.

Figure 3. (a) Graph demonstrating the density of APP positive axons in corticospinal tracts and medial lemnisci in sham injured, unsupplemented, and DHA supplemented rats. (b) Graph demonstrating the density of APP positive axons using stereologic analysis.

Figure 4.(a) Graph demonstrating the density of caspase 3 positive axons in corticospinal tracts and medial lemnisci in sham injured, unsupplemented, and DHA supplemented rats. (b) Micrograph showing caspase-3 positive axons 30 days after impact acceleration

injury.

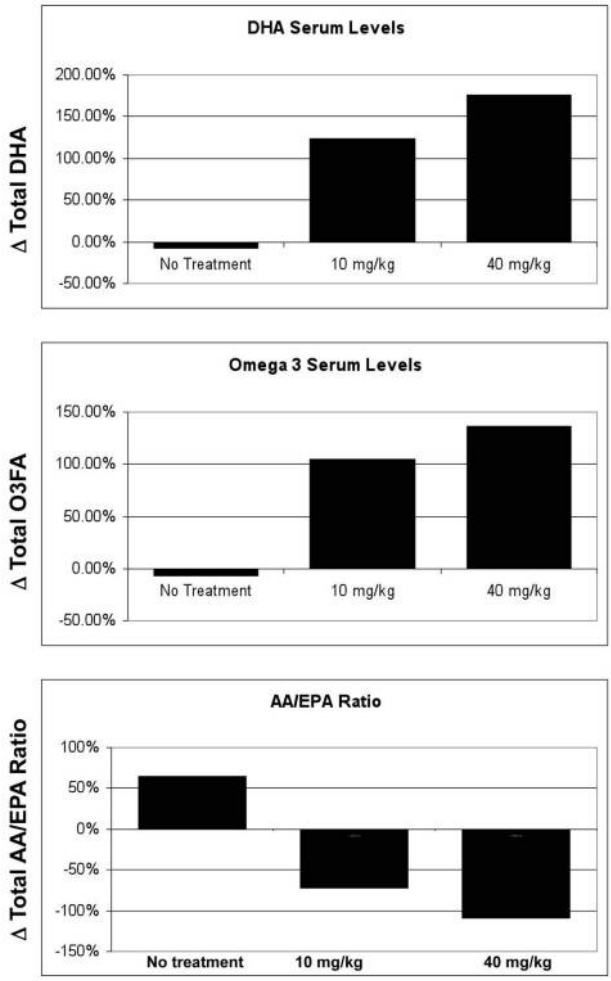

Figure 1

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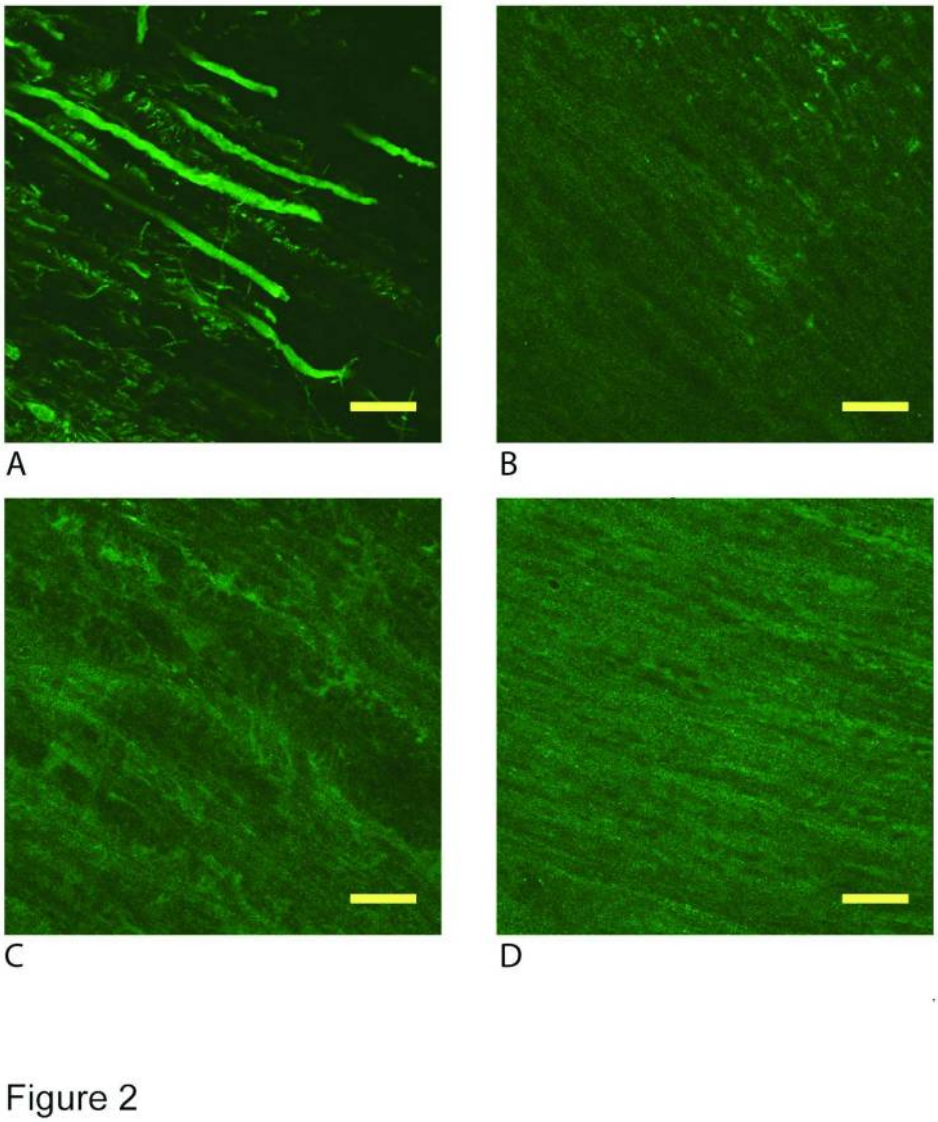

Figure 2

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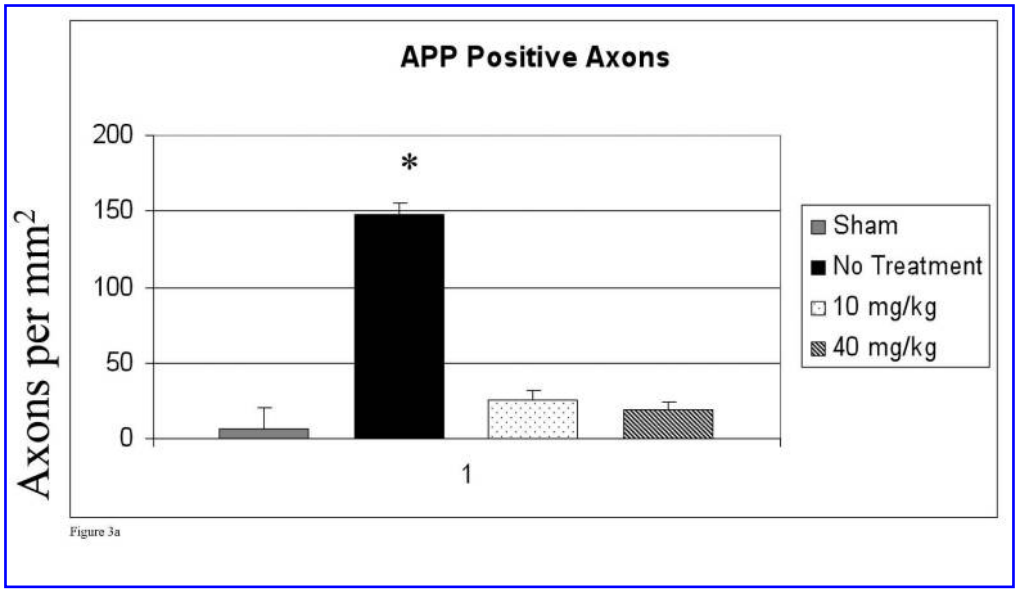

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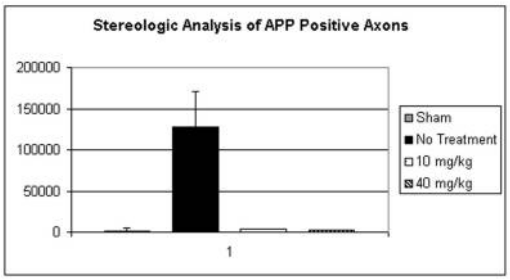

Figure 3b

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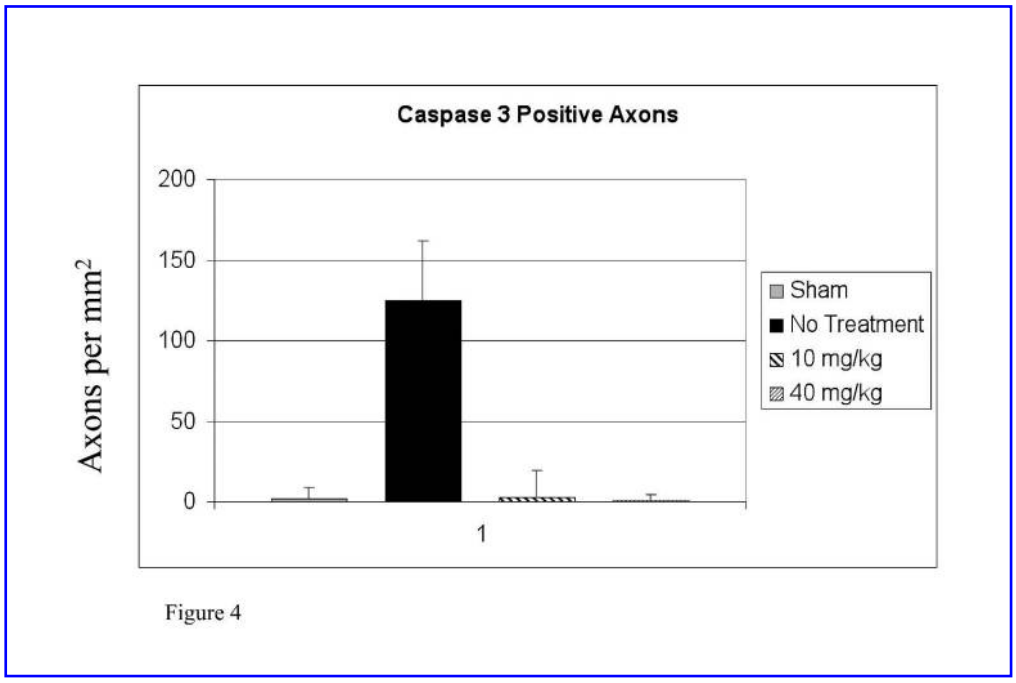

Figure 4

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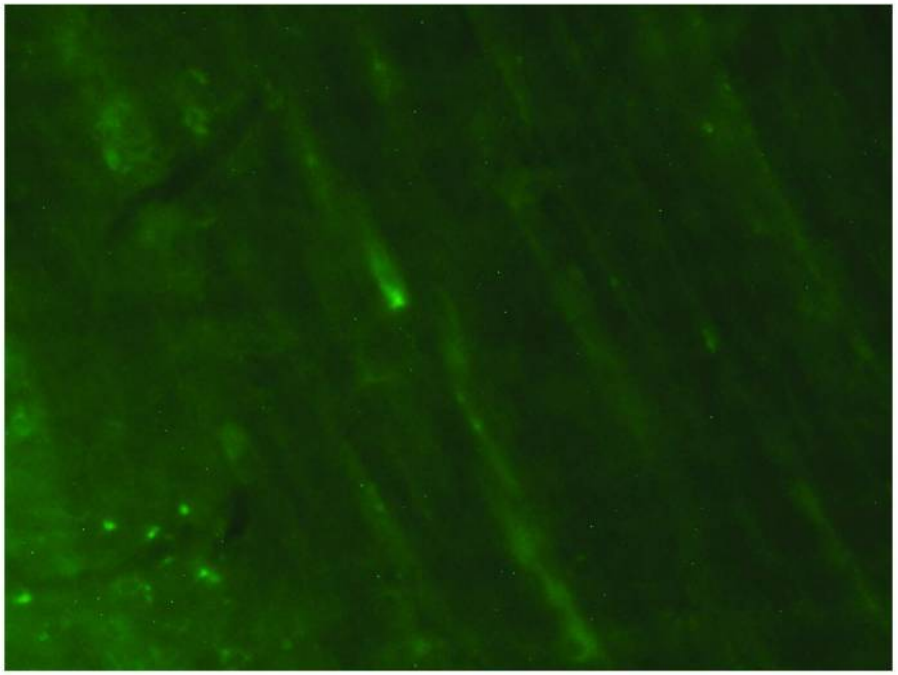

Figure 4b

152x127mm (300 x 300 DPI)