Omega-3 fatty acid supplementation and reduction of traumatic axonal injury in a rodent head injury model

Laboratory investigation

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Object. Traumatic brain injury remains the most common cause of death in persons under 45 years of age in the Western world. Recent evidence from animal studies suggests that supplementation with omega-3 fatty acid (O3FA) (particularly eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) improves functional outcomes following focal neural injury. The purpose of this study is to determine the benefits of O3FA supplementation following diffuse axonal injury in rats.

Methods. Forty adult male Sprague-Dawley rats were used. Three groups of 10 rats were subjected to an impact acceleration injury and the remaining group underwent a sham-injury procedure (surgery, but no impact injury). Two of the groups subjected to the injury were supplemented with 10 or 40 mg/kg/day of O3FA; the third injured group served as an unsupplemented control group. The sham-injured rats likewise received no O3FA supplementation. Serum fatty acid levels were determined from the isolated plasma phospholipids prior to the injury and at the end of the 30 days of supplementation. After the animals had been killed, immunohistochemical analysis of brainstem white matter tracts was performed to assess the presence of β-amyloid precursor protein (APP), a marker of axonal injury. Immunohistochemical analyses of axonal injury mechanisms—including analysis for caspase-3, a marker of apoptosis; RMO-14, a marker of neurofilament compaction; and cytochrome c, a marker of mitochondrial injury—were performed.

Results. Dietary supplementation with a fish oil concentrate rich in EPA and DHA for 30 days resulted in significant increases in O3FA serum levels: 11.6% ± 4.9% over initial levels in the 10 mg/kg/day group and 30.7% ± 3.6% in the 40 mg/kg/day group. Immunohistochemical analysis revealed significantly (p < 0.05) decreased numbers of APP-positive axons in animals receiving O3FA supplementation: 7.7 ± 4.4 axons per mm2 in the 10 mg/kg/day group and 6.2 ± 1.4 axons per mm2 in the 40 mg/kg/day group, versus 182.2 ± 44.6 axons per mm2 in unsupplemented animals. Sham-injured animals had 4.1 ± 1.3 APP-positive axons per mm2. Similarly, immunohistochemical analysis of caspase-3 expression demonstrated significant (p < 0.05) reduction in animals receiving O3FA supplementation, 18.5 ± 28.3 axons per mm2 in the 10 mg/kg/day group and 13.8 ± 18.9 axons per mm2 in the 40 mg/kg/day group, versus 129.3 ± 49.1 axons per mm2 in unsupplemented animals.

Conclusions. Dietary supplementation with a fish oil concentrate rich in the O3FAs EPA and DHA increases serum levels of these same fatty acids in a dose-response effect. Omega-3 fatty acid supplementation significantly reduces the number of APP-positive axons at 30 days postinjury to levels similar to those in uninjured animals. Omega-3 fatty acids are safe, affordable, and readily available worldwide to potentially reduce the burden of traumatic brain injury. (DOI: 10.3171/2010.5.JNS08914)

Key Words • omega-3 fatty acids • traumatic brain injury • amyloid precursor protein • dietary supplementation

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RAUMATIC brain injury remains the most common cause of death in persons under 45 years of age in the Western world. The societal impact is profound, with 2 million cases, 220,000 hospitalizations, and 52,000 deaths from head trauma occurring each year in the US alone. Another 80,000–90,000 persons each year suffer permanent debilitation. The total cost of TBI in the US, both direct health care and indirect personal and societal costs, is estimated at $44 billion per annum.30,35 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 performed in patients with injury classifications ranging from concussion to severe TBI demonstrate diffuse injury to white matter tracts running from the cortex to the brainstem. The extent of traumatic axonal injury is a principal determinant of morbidity and mortality following TBI.

Initially, traumatic axonal injury was thought to in-
volve 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 ultimate disconnection in the hours to days following TBI.\textsuperscript{24} Initial disruption of the axon plasma membrane results in ion channel dysregulation and loss of calcium homeostasis. Subsequently, a series of calcium-dependent cascades are activated, resulting in mitochondrial damage and cytochrome c release. Ultimately, cytochrome c release activates a caspase-3–mediated apoptotic cascade of proteolytic cleavage of cytoskeletal substrates resulting in the axonal disconnection characteristic of traumatic axonal injury.\textsuperscript{3,10,33} These biochemical events are related to the neurobehavioral effects of TBI, including the early onset of dementia.\textsuperscript{22}

While authors of multiple tissue and animal studies have proposed various pharmacological or physiological interventions to reduce axonal injury, to date no Phase III clinical study has shown significant effect in reducing the morbidity and mortality of TBI.\textsuperscript{13} However, a continuous series of evidence-based advances in treatment modalities used in critical care and rehabilitation medicine starting in the 1960s and continuing to the current time has greatly improved both the survival and functional outcomes of TBI patients. An improved understanding of the nutritional requirements of the critically injured patient has led to the development of both enteral and parenteral supplementation. The science of nutritional supplementation has undergone significant change from the early goals of simply delivering necessary calories to current regimens that provide specific amino acid and fatty acid combinations to maximize the healing process. Recent evidence from animal studies suggests that supplementation with O3FA (particularly EPA and DHA) improves functional outcomes following focal neural injury.\textsuperscript{36,37} It remains to be determined whether supplementation with pharmaceutical- or nutritional-grade O3FA during the initial postinjury period affects axonal injury.

We hypothesized that O3FA supplementation following diffuse axonal injury in rats would ameliorate secondary mechanisms of injury and result in fewer injured axons, as measured using immunohistochemical analysis of APP-positive axons.

**Methods**

Forty adult male Sprague-Dawley rats (weight 350–400 g) were used in this study. Three groups of 10 rats were subjected to an impact acceleration injury using the Marmarou injury model; a sham-injury procedure was performed in the remaining 10 animals (surgery as described below, but without the impact injury). Two of the groups subjected to the injury received O3FA supplementation at a dosage of 10 or 40 mg/kg/day; the third injured group served as an unsupplemented control group.

**Impact Acceleration Injury**

The Marmarou impact acceleration injury model, which results in reproducible severe TBI, was used to produce brain injuries for this study. Anesthesia was induced with intraperitoneal buprenorphine and maintained with inhaled isoflurane (using a modified medical anesthesia machine). The animals were shaved and prepared in sterile fashion for surgery, and Maracaine was injected subcutaneously into the planned incision site. A 3-cm midline incision was made in the scalp and the perietostal membranes were separated, exposing bregma and lambda.

The impact acceleration injury is performed as follows. A metal disk 10 mm in diameter and 3-mm thick is 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-g brass weight is dropped through the tube from a height of 2 m striking the disk. (This step is omitted for the sham-injury group.) The animal is then ventilated on 100% O\textsubscript{2}, while the skull is inspected and the incision repaired. When the animal recovers spontaneous respirations, anesthesia is discontinued and the animal is returned to its cage for postoperative observation.\textsuperscript{18}

All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of West Virginia University, and were 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).

**Omega-3 Fatty Acid Supplementation and Serum Level Monitoring**

The 4 groups received dietary supplementation with pharmaceutical-grade fish oil concentrate consisting of 60% of the total fatty acid quantity as EPA and DHA in a 2:1 ratio (concentrate supplied by Inflammation Research Foundation). Two of the 4 groups received dietary supplementation by gavage with a fish oil concentrate daily, starting on postinjury Day 1 (approximately 24 hours after injury), in the following amounts: Group 1, 10 mg/kg/day (6 mg/kg of EPA and DHA per day); and Group 2, 40 mg/kg/day (24 mg/kg of EPA and DHA per day). The rats in Group 3 served as unsupplemented controls; the rats in Group 4 underwent sham injury and received no supplementation. Each group received rat chow ad lib, and all animals 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 fish oil supplementation by analyzing the isolated serum phospholipids, including AA, EPA, and DHA, from 50-μl blood samples using a previously described method.\textsuperscript{13}

**Tissue Preparation and Immunohistochemical Labeling**

At 30 days postinjury all 40 animals were killed with a lethal dose injection of 0.5 ml Ketamine and 0.5 ml Xylazine. The animals were immediately perfused transcardially with 200 ml cold 0.9% saline to wash out all blood. This was followed by 4% paraformaldehyde in Millonig 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 sagittally cut on a Vibratome into 50-μm-thick sections. The tissue was subjected to temperature-controlled microwave antigen retrieval using previously described techniques and then preincubated in a solution containing 10% normal serum and 0.2% Triton X in PBS for 40 minutes.

For APP labeling, the tissue was incubated in rabbit anti–β-APP polyclonal antibody (No. 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 2 hours. Immunohistochemical markers of axonal injury and neurofilament compaction injury were analyzed using the same technique using neurofilament-M (RM014.9) mouse monoclonal antibody (No. 2838, Cell Signaling Technology, Inc.) diluted at 1:100 and secondary anti–mouse antibody conjugated with Alexa 488 (A11001, Molecular Probes). Immunohistochemical markers of active caspase-3 were analyzed using rabbit polyclonal antibody against active caspase-3 (No. 13847, Abcam) diluted at 1:200, and secondary fluorescent antibody (A11008, Molecular Probes). Immunohistochemical markers of mitochondrial dysfunction were analyzed using sheep anti–cytochrome c polyclonal antibody (No. 49879, Abcam) diluted at 1:100 in normal horse serum and secondary fluorescent antibody (A11015, Molecular Probes). The tissue was washed for the final time in 0.1-M phosphate buffer and then mounted using an antifade agent and coverslipped. The slides were sealed with acrylic and stored in the dark in a laboratory refrigerator.

Fluorescent Microscopy and Image Analysis

The tissue was examined and images were acquired using a laser scanning confocal microscope system (Zeiss) with an Argon 488 excitation laser and a 40 × objective lens. Ten digital images were obtained from the tissue of each animal, and images were then randomized. Individual injured axons were independently counted and data were stored in a spreadsheet (Microsoft Corp.). Counts were converted to density per mm² by the formula:

\[
\text{Density per mm}^2 = \frac{\text{Count}}{\text{Image area}}
\]

Differences between group means were determined using paired t-tests and considered significant if the probability value was less than 0.05.

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.

Thirty days of postinjury supplementation with concentrated fish oil containing 60% EPA and DHA at a 2:1 ratio at a dosage of either 10 mg/kg/day or 40 mg/kg/day resulted in increased levels of total serum O3FAs (C18:3ω-3, C18:4ω-3, C20:3ω-3, C20:4ω-3, C20:5ω-3, C22:5ω-3, and C22:6ω-3) of 11.6% ± 4% and 30.7% ± 3.6% over initial levels, respectively (Fig. 1). Animals receiving no supplementation had a 5.0% ± 7.4% decrease in total O3FA levels. Supplementation resulted in combined EPA and DHA serum levels of 21.8% ± 8.8% and 36.3% ± 4.1% over initial levels, respectively. Animals receiving no supplementation had an 8.9% ± 4.1% increase in combined EPA and DHA serum levels. The AA/EPA ratio, a marker of inflammation, increased 18.6% ± 9.4% in animals supplemented with 10 mg/kg O3FA per day and 17.4% ± 20.7% in animals supplemented with 40 mg/kg O3FA per day. Unsupplemented animals had an increase of 86.1% ± 19.4% in the AA/EPA ratio.

Immunohistochemical Analysis of APP-Positive Axons

In sham-injured animals, axons throughout the medullary corticospinal tract and medial lemnisci demonstrated very little labeling for APP. The rare labeled axons did not demonstrate vacuolization, swelling, or breakdown—typical characteristics of traumatic axonal injury (Fig. 2). In comparison, evaluation of axons from animals

![Graphs showing that oral supplementation with either 10 mg/kg/day or 40 mg/kg/day of concentrated fish oil for 30 days increased serum total O3FA levels (A) and combined EPA and DHA levels (B). The AA/EPA ratio, a marker of systemic inflammation, was significantly lower in animals receiving fish oil supplementation than in unsupplemented animals (C). The y axis values represent the percentage increase (or decrease) compared to preinjury values.](image-url)
that 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 lemnisci from multiple tissue slices, counting of APP positive axons was performed, and results were converted to density per mm². This demonstrated a significant quantitative difference of 182.2 ± 44.6 axons in unsupplemented animals versus sham-injured animals, which had 4.1 ± 1.3 APP-positive axons per mm² (Fig. 3).

In animals receiving either 10 mg/kg/day or 40 mg/kg/day of pharmaceutical-grade fish oil for 30 days postinjury demonstrated very few injured axons, similar to sham-injured animals (D). Bar = 20 µm.

Fig. 2. Fluorescent immunohistochemical images of brainstem sagittal sections labeled with APP antibody showing multiple swollen, disconnected axons (arrows) in corticospinal tracts and medial lemnisci in unsupplemented animals subjected to impact acceleration injury 30 days previously (A). Animals receiving either 10 mg/kg/day (B) or 40 mg/kg/day (C) of pharmaceutical-grade fish oil for 30 days postinjury demonstrated very few injured axons, similar to sham-injured animals (D). Bar = 20 µm.

Fig. 3. Graph demonstrating the density of APP-positive axons in corticospinal tracts and medial lemnisci in sham-injured, unsupplemented, and O3FA-supplemented rats. * p < 0.05, significantly different from the 3 other groups.

Discussion

This study demonstrates for the first time that a fish
Supplementation of O3FA and reduction of traumatic axonal injury

Oil concentrate rich in EPA and DHA is neuroprotective after traumatic axonal injury. Oral supplementation with either 10 mg/kg/day or 40 mg/kg/day of pharmaceutical-grade fish oil for 30 days following an impact acceleration injury resulted in significantly decreased numbers of injured axons as demonstrated by APP positivity. Likewise, a significant decrease in active caspase-3-positive axons provides additional evidence for the neuroprotective and injury-ameliorating effects of O3FAs.

Analysis of the serum phospholipid levels at the end of the postinjury supplementation period showed a doseresponse effect in the increase in the total EPA and DHA levels in the injured animals compared with the levels in the sham-injured animals, whereas there was a decrease in the combined EPA and DHA serum levels in the unsupplemented (injured) animals. Interestingly, there was a significant increase in the serum AA levels in the unsupplemented group, whereas only a nonsignificant increase was seen in the supplemented animals. As a result, the increase in AA/EPA ratio (an indicator of systemic inflammation) was significantly greater in the unsupplemented animals than in the supplemented animals. This would have the effect of activating leukocytes into neutrophils and macrophages that could more easily enter into the brain.

Several possible mechanisms for O3FA neuroprotection have been evaluated in a variety of injury model systems (Table 1). Data from spinal cord injury models demonstrates reduced apoptosis following O3FA injection in a prolonged ischemia model. More specifically, O3FA treatment has been shown to reduce oxidative stress, which in turn leads to mitochondrial dysfunction and cell death. Studies in hippocampal cells in culture demonstrate that DHA increases the activity of antioxidant enzymes, including glutathione peroxidase and glutathione reductase, as well as enhancing in vivo activity of antioxidant enzymes in the CNS. In a separate model system of forebrain ischemia, O3FAs attenuate decreases in the CNS levels of antioxidant enzymes (for example, catalase, superoxide dismutase, and glutathione peroxidase).

Modulation of the inflammatory cascade by O3FAs 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 proinflammatory eicosanoids, such as 2-series prostaglandins, thromboxanes, and leukotriene B4. 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, and interleukin-6. In contrast, O3FAs can decrease COX activity and inhibit the formation of proinflammatory eicosanoids and cytokines.

Although there are low levels of EPA in the brain, its increased levels in the blood and peripheral tissue would provide the precursor of the weakly antiinflammatory series-3 prostaglandins and also inhibit conversion of AA to proinflammatory prostaglandins and leukotrienes external to the brain. In a spinal cord injury model, 20-fold increases in tissue levels of AA have been demonstrated, resulting in recruitment of neutrophils, macrophages, and microglial cells to the injury site.

Additional mechanisms through which O3FAs may play a neuroprotective role include reduction in excitotoxicity, modulation of calcium and potassium channels, activation of gene transcription, formation of neuroprotectin-1 and resolvins (Fig. 5). In vitro and in vivo studies have shown that O3FAs reverse glutamate-induced excitotoxicity, a known trigger of apoptosis after spinal cord injury. Omega-3 fatty acids have significant affinity for 2-pore potassium channels, such as TWIK-related potassium channel (TREK) and TWIK-related AA-stimulated potassium channel (TRAAK). Both EPA and DHA have also been demonstrated to inhibit calcium channels. This could potentially prevent apoptosis of damaged neurons and their projection fibers. The neuroprotective effect of DHA may involve activation of gene transcription.
**TABLE 1: Proposed mechanisms of O3FAs**

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<td>increased glutathione peroxidase &amp; glutathione reductase activity</td>
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<td>antiinflammatory</td>
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<td>inhibition of downstream JNK pathway</td>
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<td>platelet assay</td>
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<td>reduction in excitotoxicity</td>
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<td>neurogenesis</td>
<td>cell culture</td>
<td>DHA</td>
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<td>Calderon &amp; Kim, 2004</td>
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**Fig. 5.** Artist’s illustration showing the mechanisms by which O3FAs may play a neuroprotective role after TBI.
through retinoid receptor signaling. DHA can lead to the formation of mediators such as 10,17S-docosatriene (neuroprotectin D1), an endogenous compound with antioxidant effects as well as antiinflammatory effects. EPA increases the levels of resolvins, thus further decreasing the intensity of the inflammatory process. In hippocampal neurons, O3FAs have neurite growth-promoting effects. A recent finding shows reduction in the levels of 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 neuron survival.

The numerous proposed mechanisms of action of O3FAs 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 and modulate continued energy production. Our findings are consistent with previous research that has demonstrated that O3FAs are protective against cellular injury. Furthermore, this study demonstrates that O3FAs ameliorate axonal injury when administered following injury, a finding of clinical importance not previously reported.

Omega-3 fatty acid preparations have been previously shown to be safe and well tolerated by patients in several diseases. Lipid emulsions are already given as nutritional support to a variety of patients in critical care settings. Ultra-refined fish oil concentrates containing high quantities of EPA and DHA are commercially available, increasing the ease with which they could be translated into clinical use in TBI. Administration of O3FAs after brain trauma as a neuroprotective and injury-ameliorating treatment deserves consideration and further clinical investigation as a promising and innovative approach in TBI management.

Conclusions

Dietary supplementation with O3FAs increases serum levels of these same fatty acids. Omega-3 fatty acid supplementation significantly reduces the number of APP-positive axons at 30 days postinjury to levels similar to those in uninjured animals. Omega-3 fatty acids are safe, affordable, and readily available worldwide to potentially reduce the burden of TBI.

Disclosure

Dr. Sears is the founder and trustee of the Inflammation Research Foundation, which provided fish oil concentrate and material support for this project. No other authors have a financial interest in the materials presented in this paper.

All authors reviewed the final version of the manuscript and approved it for submission.

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