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FISH OIL IMPROVES MOTOR FUNCTION, LIMITS BLOOD-BRAIN BARRIER DISRUPTION, AND REDUCES MMP9 GENE EXPRESSION IN A RAT MODEL OF JUVENILE TRAUMATIC BRAIN INJURY

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SUMMARY

The effects of an oral fish oil treatment regimen on sensorimotor, blood-brain barrier, and biochemical outcomes of traumatic brain injury (TBI) were investigated in a juvenile rat model. Seventeen-day old Long-Evans rats were given a 15 mL/kg fish oil (2.01 g/kg EPA, 1.34 g/kg DHA) or soybean oil dose via oral gavage 30 minutes prior to being subjected to a controlled cortical impact injury or sham surgery, followed by daily doses for seven days. Fish oil treatment resulted in less severe hindlimb deficits after TBI as assessed with the beam walk test, decreased cerebral IgG infiltration, and decreased TBI-induced expression of the *Mmp9h* gene one day after injury. These results indicate that fish oil improved functional outcome after TBI resulting, at least in part from decreased disruption of the blood-brain barrier through a mechanism that includes attenuation of TBI-induced expression of *Mmp9*.

Keywords

traumatic brain injury; juvenile; rat; fish oil; blood-brain barrier; matrix metalloproteinase 9

INTRODUCTION

Traumatic brain injury (TBI) is a major cause of death and acquired disability in young children [1]. Despite having a high degree of neuroplasticity, young children tend to have poorer outcomes after TBI than adults [2]. Children can also exhibit differential responses to pharmacological interventions including altered bioavailability, metabolism, and drug response [3, 4]. It is thus critical that both the effect of TBI and potential therapeutics be investigated in an age-appropriate model [5].

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The n-3 long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA, 20:6n-3) and docosapentaenoic acid (DHA, 22:6n-3), the main constituents in fish oil, are biologically active with many neuroprotective properties. When consumed in the diet or via supplementation, these n-3 fatty acids are incorporated into the phospholipids that form cell membranes where they can alter the physicochemical and membrane-signaling properties of the cell [6, 7]. DHA, EPA and their metabolites also have anti-excitotoxic [8], antioxidant [9], anti-apoptotic [10, 11], and anti-inflammatory properties [12]. During development, N-3 fatty acids, particularly DHA, accumulate in the brain during late gestation and early post-neonatal life in humans and rats, a time at which children have a high risk for sustaining traumatic brain injuries [13–15]. Additionally, EPA and DHA can be metabolized into several families of molecules including NPD1, docosanoids, resolvins, *etc.*, which have been shown to be neuroprotective through their anti-inflammatory and inflammation-resolving activities [16–18]. LC-PUFA can also directly or indirectly modulate gene expression through activation or suppression of cell signaling pathways and transcription factors (e.g., PI3K/Akt, NF- κ B, PPAR and RXR) [19–21].

DHA and EPA, the primary active constituents in fish oil, readily cross the blood-brain barrier [22]. Studies of various neural injury models including TBI and spinal cord injuries in adult animals indicate that fish oil, or DHA or EPA alone produce beneficial effects [23–25]. Furthermore, in a case report, high dose fish oil supplementation (19.2 g/day) was associated with substantial clinical improvement in a young patient with severe, potentially lethal, head trauma [26]. However, the effects of LC-PUFA or fish oil treatment have not been investigated in juvenile brain injury. Accordingly, this study investigated the use of oral fish oil treatment in a juvenile rat model on sensorimotor and biochemical outcomes of TBI, including blood-brain barrier disruption and levels of *Ccl2*, *Gfap*, and *Mmp9* mRNAs, representative key mediators involved in immune cell recruitment, astrogliosis, and blood-brain barrier disruption after TBI. We will show that fish oil resulted in improved functional outcome, at least in part, by limiting disruption of the blood-brain barrier through a mechanism that includes attenuation of TBI-induced expression of *Mmp9*.

MATERIALS AND METHODS

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Animals, husbandry, and treatment

Long-Evans rats were housed in a temperature- and humidity-controlled facility with a 14–10 hour light-dark cycle (on at 06:00 h) with *ad libitum* access to water and chow (Teklad Global diet 2016, Harlan Laboratories, Inc., Indianapolis, IN). The chow contained 4% fat by weight and was formulated with soybean oil resulting in a fatty acid composition of 0.5% 16:0, 0.1% 18:0, 0.7% 18:1n-9, 2% 18:2n-6, 0.1% 18:3n-3. Breeding stock (females 75–85 days; male proven breeders; Harlan Laboratories, Inc. Indianapolis, IN) were obtained a minimum of 5 days prior to the beginning of the experiment and were handled regularly. Litters were culled to eight pups with preference for males on postnatal day (PND) one. Male pups (n = 4–12/group, depending on endpoint, and each from a different litter) received either a controlled cortical impact (CCI) injury or sham surgery on PND 17 and were returned to the dam until weaning. Pups were weaned on PND 20 and housed in groups of two to four, TBI and sham-injured together, for the remainder of the study.

Rats were treated with either 15 mL/kg of fish oil (2.01 g/kg EPA, 1.34 g/kg DHA; Nature Made 1200 mg fish oil capsules, Mission Hills, CA) or unhydrogenated soybean oil via oral gavage 30 minutes prior to the initial TBI or Sham surgery, and then daily for seven days.

On days behavioral testing occurred (see below), oil was administered after testing to avoid any confounding effect the gavage procedure might have on behavior. On the day of euthanasia, rats were decapitated without anesthesia approximately six hours after oil administration. Soybean oil was used as the comparator in this study to control for the caloric content of the fish oil, and, because it is the oil used in the formulation of the Teklad Global #2016, it does not introduce any fatty acids not already consumed by rats in both treatment groups. The fatty acid composition of the oils is shown in Table 1.

Rats used for assessment of sensorimotor function were tested 1, 4, and 7 days after surgery and then euthanized on day 7 by transcardial perfusion under pentobarbital anesthesia, followed by removal of the brain for IgG immunohistochemistry. Rats used for determination of mRNA levels were euthanized on day 1 (28 hrs after surgery) or day 4 after surgery by decapitation. Brains from these rats were rapidly removed and then dissected on ice. The frontal cortex, a cortical region remote from the contusion site, which was not needed for mRNA analyses and has a fatty acid composition representative of whole brain [27], was frozen on dry ice for later fatty acid analysis. The injured motor cortex was preserved in RNAlater (Life Technologies/Ambion, Gaithersburg, MD) for mRNA analysis.

Controlled cortical Impact

CCI was performed as previously described [28, 29]. Briefly, rats were stabilized in a Cunningham stereotaxic frame (Stoelting, Wood Dale, IN) after being anesthetized with isoflurane (induction, 3.0%; maintenance, 2.0%). A 4×4 mm craniotomy was performed lateral (right side) to the mid-sagittal suture, centered at: AP = 0, ML = 2.5 from bregma. The impactor device, previously described in detail [30] was equipped with a 3.0 mm-diameter tip. The impactor tip was centered within the craniotomy and lowered until the tip just contacted the dura over motor (M1, M2) and sensory (S1FL, S1HL) cortical areas [31, 32]. The parameters of the impact were as follows: 3.0 mm depth, 1.5 m/sec strike velocity, 300 msec contact time. The scalp incision was then closed with a 6-0 silk suture and the animal was allowed to recover from anesthesia.

Sham procedures involving the use of a trephine or drill to produce craniotomy have been shown to cause brain injury distinct from that caused by the impact [33, 34]. Though cortical damage caused by TBI significantly outweighs damage produced by the craniotomy [35], the sham surgery consisted of an incision in the scalp with no craniotomy or impact from the CCI device to avoid potential experimental confounds that might be caused by any additional intervention.

All rats received 0.05 mg/kg of buprenorphine approximately thirty minutes after surgery and again 24 hours after surgery, after day 1 behavioral testing was completed. The administration of buprenorphine analgesia after CCI surgery was required by the Institutional Animal Care and Use Committee. Buprenorphine is a μ -opioid receptor partial agonist and is thus analgesic, but not anti-inflammatory [36]. Opioid analgesics are clinically contraindicated for use in TBI because they can increase intracranial pressure thus exacerbating the effects of injury; however, the craniotomy required for the CCI likely minimizes this effect. Furthermore, the administration of buprenorphine to all treatments groups ensures that any effects it may produce are controlled for.

Beam walk test

Rats were tested for their ability to traverse a 75 cm-long wooden dowel (15 mm diameter) elevated 30 cm and ending in a dark goal box as previously described in detail [29]. Video-taped sessions were scored for ipsilateral and contralateral foot slips, time required to reach the goal box, and total number of steps taken by the right (uninjured) hind foot. Data are

reported as the percent of contralateral foot slips relative to the total number of steps needed to traverse the beam.

Brain Total Phospholipid Fatty Acid Composition

Brain total phospholipid fatty acid composition was analyzed as previously described [27]. Briefly, phospholipids were extracted from frontal cortex and isolated by thin layer chromatography. The phospholipids were then transmethylated with boron trifluoride methanol (Sigma, St. Louis, MO) to produce fatty acid methyl esters. Individual fatty acid methyl esters were analyzed using a Varian 3400 gas chromatograph with an SP-2330 capillary column (30 m, Supelco, Inc., Belfonte, PA), using helium as the carrier gas. Peaks were identified by comparing to authentic standards (PUFA 1 and 2, and Supelco 37, Supelco, Inc. and 22:5n-6, Nu-Chek Prep, Elysian, MN) and corrected for response factors. Individual fatty acids were expressed as weight percent of total fatty acids on the basis of peak area.

IgG Immunohistochemistry

Frozen coronal sections (30 μ m) through the site of impact (+2.0 mm to -2.0 mm bregma), or the corresponding area in shams, collecting every tenth section, and mounted on Fisherbrand Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were stained for IgG using a Vectastain Rat IgG ABC Kit (Vector Labs, Burlingame, CA) according to the manufacturer's protocol. IgG immunoreactivity was visualized using ABC (streptavidin-HRP) and diaminobenzidine tetrahydrochloride (Vector Labs, Burlingame, CA). ImageJ [37] was used to determine the ipsilateral cortical IgG staining density and total area of IgG staining (both hemispheres) from macro-level digitized grayscale images of each section. The total area of IgG staining was calculated from IgG staining in the entirety of both hemispheres. A baseline grayscale value threshold was set at 75 based on the background level of nonspecific staining in sham sections. Any areas darker than the threshold were considered positive for IgG staining. The volume of IgG staining was calculated using the following equation: IgG area * section thickness * distance between sections = Subvolume. Total IgG Volume = Σ Subvolume (section₁ + section₂ + ...section_n).

Quantitative Real-Time PCR

Quantitative real-time PCR was performed as previously described in detail [28]. Total RNA was isolated using a Trizol (Life Technologies/Ambion) phenol-chloroform extraction according to the manufacturer's protocol and precipitated overnight with 75% isopropyl alcohol. The quality of isolated RNA quality was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) with adequate quality being an OD 260/280 greater than 1.8. mRNA quality was further verified using a Agilent Bioanalyzer 2011 (Agilent Technologies, Inc., Santa Clara, CA). First strand cDNAs were synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) per the manufacturer's protocol using a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA). Exon spanning, gene-specific primers (Supplemental Table 1) were prepared using the NCBI's Primer-BLAST [38] and purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Primer specificity was determined by the presence of a single peak in the melt curve. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method [39]. Data are expressed as fold change in gene expression compared to the reference gene beta-2-microglobulin (*B2m*), which was experimentally determined to be the most stably expressing gene in our experimental model and brain region of interest [40].

Statistical Analysis

All data are expressed at the mean $\pm$ SEM. Data were analyzed for effects of injury (TBI or Sham-injured) and oil (Soybean oil or Fish oil) by ANOVA with factors of TBI, oil, and day after injury (1–7 days after injury) (Systat, v.12). Time after injury was analyzed as repeated measure for the sensorimotor function studies. Outliers identified by Systat were discarded from subsequent analyses. Post-hoc comparisons were made using 1-way ANOVA and Fisher's Least Significant Difference test, or Student's t test. Differences were considered significant if $P < 0.05$.

RESULTS

Effects on Growth, Development, and Brain Phospholipid Fatty Acid Composition

Overall growth and development of the rats was not affected by TBI or oil treatment (Figure 1).

Total phospholipid fatty acid composition was assessed in the frontal cortex of each brain four days after injury (Table 2). In Sham-injured rats, administration of fish oil reduced the percentage of the monounsaturated fatty acid (MUFA) 24:1 and Other MUFA by 16% ($P < 0.05$) and 57% ($P < 0.05$), respectively, compared to soybean oil. Fish oil administration also significantly reduced the Other n-6 and 17% ($P < 0.01$) compared to soybean oil-treated shams.

TBI resulted in a significant main effect of decreased Total PUFA ($P < 0.05$), Total n-3 ($P < 0.05$) and Total n-6 ($P < 0.05$), resulting from significant decreases in 22:6n-3 ($P < 0.05$), 20:4n-6 ($P < 0.05$) and 22:5n-6 ($P < 0.05$), and an increase in Total SFA ($P < 0.01$); however, these effects were not quite statistically significant in post hoc comparisons ($P = 0.05 - 0.09$).

Effects on Sensorimotor Function

Sensorimotor function was altered in all rats that sustained a TBI as indicated by an overall significant increase in the percentage of unilateral beam walk slips (Figure 2), indicating deficits in hindlimb function [41]. Repeated-measures ANOVA indicated that rats treated with either oil exhibited significant improvement from day one to day seven; however, post-hoc analysis indicated that rats treated with fish oil had less functional deficit on days one and seven after injury than those treated with soybean oil ($P < 0.05$).

Effects on IgG staining

Rats with TBI exhibited extensive IgG staining seven days after TBI. There was no significant difference in the density of IgG staining between the injured rats in both oil treatment groups (not shown). There was, however, a significant interaction with the oil treatments such that IgG infiltrated a smaller volume of brain area in rats treated with fish oil than with soybean oil ($P < 0.05$) (Figure 3). The volume of IgG stained tissue was not different in sham-injured rats treated with either oil (not shown).

Effects on TBI-induced mRNA levels

Chemokine CC ligand-2 (*Ccl2*) mRNA level was increased on day 1 after TBI approximately 35-fold ($P < 0.05$ v. Sham) in rats treated with soybean oil. A non-significant increase in *Ccl2* mRNA level of approximately 15-fold was also observed in rats with TBI treated with fish oil ($P = 0.091$ v. Sham) (Figure 4A). In rats treated with fish oil, the TBI-induced increase in *Ccl2* mRNA level returned to near-baseline levels on day 4 after TBI,

but were still significantly increased in the TBI soybean oil group compared to soybean oil-treated sham controls ($P < 0.01$).

Glial fibrillary acidic protein (*Gfap*) mRNA level was also increased by TBI by 10-fold on day 1 after TBI in the soybean oil-treated group ($P < 0.05$ v. Sham). An 8-fold increase was also observed in the fish oil treated group, but this was not quite significant and was not different from the increase observed in soybean oil-treated rats ($P = 0.053$ v. Sham) (Figure 4B). *Gfap* mRNA level decreased to 3–4-fold on day 4 after TBI ($P < 0.05$ v. Sham) and was not different between soybean oil- and fish oil-treated rats.

Matrix metalloproteinase-9 (*Mmp9*) mRNA level was increased on day 1 after TBI approximately 2-fold in rats treated with soybean oil ($P < 0.05$) (Figure 4C). In contrast, mRNA level for *Mmp9* was not increased on day 1 after TBI in rats treated with fish oil and was significantly different from those treated with soybean oil ($P < 0.05$). On day 4 after surgery, *Mmp9* mRNA level in rats with TBI was lower than in Sham-injured rats in both soybean oil- and fish oil-treated rats ($P < 0.001$). In addition, there was an effect of fish oil in the Sham-injured rats such that levels of *Mmp9* mRNA were 50% higher in Sham-injured rats treated with fish oil than in those treated with soybean oil ($P < 0.01$).

DISCUSSION AND CONCLUSIONS

LC-PUFA or fish oil treatment has been beneficial in treating adult neural injuries in several models, including humans [24–26, 42–44]. This study investigated the effects of oral fish oil treatment in 17-day old rats, which are comparable to human toddlers with respect to motor development [45, 46], on the functional and molecular outcomes of TBI. The treatment paradigm used, with oil treatment beginning 30 min prior to injury, was designed to ensure increased levels of n-3 LC-PUFA during all early phases of the events triggered by TBI to best test the hypothesis that fish oil mitigates the effects of TBI, rather than model a clinical treatment scenario.

Injury to the primary motor cortex of 17 day-old rats produced deficits in hindlimb function as assessed using the beam walk test (Figure 2), a sensorimotor test previously validated for use with this CCI model of juvenile TBI (Russell et al. 2011). Consistent with previous studies [47, 48], TBI also increased expression of the *Ccl2* and *Gfap* genes (Figure 4), mediators involved in immune cell recruitment and astrogliosis, respectively, and *Mmp9*, a mediator of blood-brain barrier disruption [28, 49]. Also in agreement with previous studies [50, 51], TBI caused significant infiltration of IgG, a serum protein, into the brain parenchyma (Figure 3), indicating disruption of the blood-brain barrier [52]. Together, these data indicate that an injury specific to the sensorimotor cortex with persisting functional deficits was achieved.

In addition to the effects of TBI on sensorimotor function and gene expression, TBI significantly altered the fatty acid composition of the frontal cortex. Four days after injury, TBI resulted in a main effect of decreased percentage of LC-PUFA in brain membranes, which was accompanied by an increase in saturated fatty acids (Table 2). Because this study used semi-quantitative methods which assess the fatty acid composition of brain phospholipids, rather than the absolute amounts of each fatty acid, these changes could be due to substitution of saturated fatty acids for LC-PUFA or changes in the absolute amounts of specific fatty acids. However, other studies indicate that TBI causes a rapid, sustained increase of free fatty acids [53], including DHA (Chris Butt, personal communication). Therefore, it is likely that the cleavage of n-6 and n-3 fatty acids from the membrane, as a result of TBI, is responsible for the observed decrease in the total percentage of membrane LC-PUFAs.

In sham-injured rats, administration of either fish oil or soybean oil did not cause any alterations in growth (Figure 1), behavior (Figure 2), IgG infiltration into the CNS (Figure 3), or expression of the *Ccl2* or *Gfap*, genes (Figure 4). This indicates that soybean oil and fish oil administration was well-tolerated. Interestingly, fish oil increased *Mmp9* mRNA level in sham-injured rats after 4 days of administration (Figure 4), the physiological importance of which must be determined in future studies.

In rats with a CCI injury, fish oil administration decreased the magnitude and persistence of TBI-induced motor function deficits, reduced the extent of IgG infiltration into the brain parenchyma (Figure 3), and produced some reduction in TBI-induced *Gfap* gene expression (Figure 4B) when compared to rats treated with soybean oil. Furthermore, fish oil treatment prevented the early TBI-induced increase in *Mmp9* mRNA level (Figure 4C), a key mediator in the breakdown of the blood-brain barrier, and attenuated the TBI-induced decrease in brain DHA (Table 2) that was observed in soybean oil-treated rats. Beneficial effects of dietary supplementation with n-3 LC-PUFA have been reported in studies using adult models of TBI on end points such as TBI-induced apoptosis and deficits in the Morris water maze. [23, 42, 44, 54–57]. In other models of neural injury such as ischemia-reperfusion, injections of DHA decreased blood-brain barrier permeability [24], as well as improving injury-induced deficits in neurological score and infarct volume [58, 59]. Furthermore, DHA and EPA have been shown to decrease protein levels and activity of MMP-9 [60], suggesting that the changes in *Mmp9* mRNA observed in this study would be consistent with decreased MMP-9 activity, and thus decreased disruption of the blood-brain barrier. Thus, the present findings generally agree with the literature and support the use of fish oil as a treatment for TBI in both juveniles and adults.

The present findings also suggest that n-3 LC-PUFA in fish oil enhance outcomes after TBI through a mechanism that includes limiting blood-brain barrier damage after TBI and/or expediting its repair. The blood-brain barrier is a dynamic, complex structure made up of vascular endothelial cells surrounded by support cells and astrocytic foot processes [61]. A growing body of evidence implicates MMP-9 in the breakdown of the blood-brain barrier after neural injury in both juvenile and adult animal models [62–66]. MMP-9 disrupts the blood-brain barrier by degrading collagen IV and laminin in the basal lamina [67], as well as MMP-dependent cleavage of blood-brain barrier tight junction proteins leading to significant disruption of cell-cell contact [68].

Mmp9 is regulated at the level of gene transcription, primarily through an NF- κ B site in the promoter region [69]. LC-PUFA are known to regulate NF- κ B signaling through binding to and activating PPAR receptors thereby antagonizing the NF- κ B signaling pathway [70] or through directly inhibiting activation of NF- κ B independently of PPAR [21, 71]. From this we can hypothesize that fish oil may limit gene expression of *Mmp9* in activated astrocytes, microglia, and perhaps other cells, through inhibition of NF- κ B signaling early after injury; however, this must be determined in future studies.

In conclusion, the present data show that treatment with fish oil improved TBI outcomes in juvenile rats, resulting, at least in part, from decreased disruption of the blood-brain barrier through a mechanism that included attenuation TBI-induced expression of *Mmp9*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effects of injury and fish oil treatment on body weight

Data are the mean $\pm$ SEM ($n = 11-12$ per group). Rate of weight gain did was not altered by TBI or oil treatments as assessed by repeated-measures ANOVA.

Figure 2. Effects of fish oil treatment of TBI-induced hindlimb deficits assessed using the beam walk test. Data are the mean $\pm$ SEM ($n = 11-12$ per group). * $P < 0.05$ vs. same day, different oil; † $P < 0.05$ vs. same oil, day one by repeated-measures ANOVA and Fisher's LSD test.

Figure 3. Effects of fish oil treatment on TBI-induced cerebral IgG Infiltration
Data are the mean $\pm$ SEM (n = 10–11 per group). *P < 0.05 by Student's-t test.

Figure 4. Effects of fish oil on TBI-induced changes in *Ccl2* (A), *Gfap* (B), and *Mmp9* (C) mRNA levels
 Data are the mean $\pm$ SEM (n = 4–7 per group). *P < 0.05 by ANOVA and Fisher's LSD test.

Table 1

Fish and soybean oil fatty acid composition.

Percent of Total Fatty Acids (wt%)		
Fatty Acid	Fish Oil	Soybean Oil
14:0	7.73	ND
16:0	15.95	11.12
16:1	13.63	0.10
18:0	5.18	5.05
18:1n-9c	10.26	20.99
18:2n-6c	3.63	50.10
18:3n-6	0.45	ND
18:3n-3	0.77	9.74
20:1n-9	1.04	0.13
20:2n-6	0.37	0.32
20:3n-6	0.23	ND
20:4n-6	1.70	0.02
22:1	0.22	0.04
20:5n-3	11.57	ND
22:2n-6	0.13	0.27
22:5n-6	0.36	ND
22:6n-3	16.39	ND

ND: not detected

Table 2

Effects of TBI and fish or soybean oil treatment on total phospholipid fatty acid composition of frontal cortex four days after initiation of treatment with fish oil or soybean oil.

Fatty Acid	Percent of Total Fatty Acids (wt%)			
	Soybean Oil		Fish Oil	
	Sham	TBI	Sham	TBI
16:0	24.14 ± 0.85	27.27 ± 1.07	26.90 ± 1.66	25.72 ± 0.73
18:0	23.07 ± 0.67	23.16 ± 0.76	17.98 ± 3.99	24.48 ± 0.76
Other SFA	1.42 ± 0.16	2.60 ± 0.34	2.32 ± 0.53	2.02 ± 0.31
18:1n9	9.17 ± 1.67	10.17 ± 1.27	11.46 ± 0.77	10.13 ± 0.51
24:1	4.74 ± 0.25	4.04 ± 0.26	3.99 ± 0.22 ^b	3.43 ± 0.20 ^b
Other MUFA	2.27 ± 0.12	1.44 ± 0.26 ^a	0.97 ± 0.17 ^b	1.58 ± 0.35
20:4n6	14.60 ± 0.41	13.57 ± 0.56	15.26 ± 0.79	13.39 ± 0.44
22:5n6	1.97 ± 0.16	1.66 ± 0.10	1.64 ± 0.10	1.40 ± 0.10 ^b
Other n-6	2.28 ± 0.07	2.28 ± 0.06	1.75 ± 0.17 ^b	1.75 ± 0.21 ^{bc}
22:6n3	17.09 ± 1.72	13.98 ± 0.70	18.49 ± 1.20	15.86 ± 0.83
Other n-3	0.08 ± 0.04	0.05 ± 0.03	0.06 ± 0.01	0.05 ± 0.01
ΣSFA	48.64 ± 1.61	53.02 ± 1.59	47.20 ± 2.11	52.21 ± 0.66
ΣMUFA	16.18 ± 1.47	15.65 ± 1.50	16.42 ± 0.99	15.14 ± 0.42
Σn-6	18.85 ± 0.56	17.51 ± 0.59	18.64 ± 0.92	16.54 ± 0.47
Σn-3	17.17 ± 1.74	14.05 ± 0.70	18.55 ± 1.20	15.92 ± 0.83
ΣPUFA	36.02 ± 2.17	31.54 ± 1.21	37.20 ± 1.95	32.46 ± 1.05

Other saturated fatty acids (SFA): 13:0, 14:0, 15:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; Other monounsaturated fatty acids (MUFA): 15:1, 16:1, 20:1n-9; Other n-6: 18:2n-6c, 18:3n-6, 20:2n-6, 20:3n-6, 22:2n-6; Other n-3: 18:3n-3, 20:5n-3. Data are presented as the mean ± SEM (n = 6-7 per group selected at random from the total sample size).

^aP < 0.05 vs. Sham, Same Oil;

^bP < 0.05 vs. Sham, Different Oil;

^cP < 0.05 vs. TBI, Different Oil by ANOVA and Fisher's LSD test.

Two-way ANOVA indicated that TBI produced a significant main effect of decreased Total PUFA (P < 0.05), Total n-3 (P < 0.05) and Total n-6 (P < 0.05), resulting from significant decreases in 22:6n-3 (P < 0.05), 20:4n-6 (P < 0.05) and 22:5n-6 (P < 0.05), and an increase in Total SFA (P < 0.01); however, these effects were not quite statistically significant in post hoc comparisons (P = 0.05 – 0.09).