Omega-3 polyunsaturated fatty acids protect neurological function after traumatic brain injury by suppressing microglial transformation to the proinflammatory phenotype and activating exosomal NGF/TrkA signaling

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Research Article

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Abstract

The transformation of microglia to a pro-inflammatory phenotype at the site of traumatic brain injury (TBI) drives the progression of secondary neurodegeneration and irreversible neurological impairment. Omega-3 polyunsaturated fatty acids (PUFAs) have been shown to suppress this phenotype transformation, thereby reducing neuroinflammation following TBI, but the molecular mechanisms are unknown. We found that Omega-3 PUFA suppressed the expression of disintegrin metalloproteinase (ADAM17), the enzyme required to convert tumor necrosis factor-a (TNF-α) to the soluble form, thereby inhibiting the TNF-α/NF-κB pathway both in vitro and in a mouse model of TBI. Omega-3 PUFA also prevented the reactive transformation of microglia and promoted the secretion of microglial exosomes containing nerve growth factor (NGF), activating the neuroprotective NGF/TrkA pathway both in culture and TBI model mice. Moreover, Omega-3 PUFA suppressed the pro-apoptotic NGF/P75NTR pathway at the TBI site and reduced apoptotic neuronal death, brain edema, and disruption of the blood–brain barrier. Finally, Omega-3 PUFA preserved sensory and motor function as assessed by two broad-spectrum test batteries. The beneficial effects of Omega-3 PUFA were blocked by an ADAM17 promotor and by a NGF inhibitor, confirming the pathogenic function of ADAM17 and the central neuroprotective role of NGF. Collectively, these findings provide a strong experimental basis for Omega-3 PUFA as a potential clinical treatment for TBI.

Introduction

Traumatic brain injury (TBI) caused by an external force to the head is a major cause of death and permanent disability[1–3]. Direct mechanical damage and secondary pathological processes such as excitotoxicity, oxidative stress, and mitochondrial dysfunction can promote the secretion of inflammatory mediators, leading to further free radicals generation and a sustained inflammatory cascade that further amplifies the extent of injury. Therefore, effective control of neuroinflammation after TBI can help facilitate neuronal survival and functional recovery [4].

Microglia are the resident macrophages in the central nervous system, and account for 5–15% of all neuroglia in the brain [5]. Microglia are divided into a pro-inflammatory phenotype and an anti-inflammatory phenotype that co-exist within the TBI area and both are engaged in modulating the neuroinflammatory response. Pro-inflammatory microglia secrete inflammatory factors that act on adjacent cells, perpetuating brain tissue damage and impairing function [6]. Tumor necrosis factor-a (TNF-α) is the main inflammatory mediator secreted by microglia after TBI. It is secreted as a precursor that must be cleaved by extracellular A disintegrin metalloproteinase 17 (ADAM17) to initiate inflammatory responses as soluble (s)-TNF-α [7], and inhibition of ADAM17 can effectively reduce TNF-α production induced by lipopolysaccharide (LPS) [8]. Soluble TNF-α binding to its receptor triggers the translocation of nuclear factor-κB (NF-κB), resulting in the activation of genes programs and pathways that regulate microglia phenotype and the ensuing inflammatory cascade [9–12], including the secretion and release of factors such as interleukin (IL)-1, IL-6, and nitric oxide that sustain the inflammatory process[13, 14].
These is mounting evidence that cells communicate through the release and uptake of small lipid exosomes containing various signaling factors, including proteins and nucleic acids [15] that initiate neuroinflammatory and neuroprotective cascades after TBI [16, 17]. Different microglial phenotypes secrete exosomes containing substances with distinct effects on neuroinflammation and neurological function after TBI [18]. For instance, pro-inflammatory microglia can inhibit synaptogenesis and induce neuronal apoptosis through the release of exosomes containing microRNA-146a and microRNA-383-3p [19, 20], while anti-inflammatory microglia secrete exosomes that promote neuronal production of ceramide and sphingomyelin to modulate synapses, improve excitatory transmission, and prevent neurological dysfunction [20, 21]. Therefore, it is important to explore the mechanisms of microglial phenotypic regulation and the distinct exosome populations contributing to exacerbation or reduction of neuroinflammation and neurological impairment after TBI [22].

Polyunsaturated fatty acids (PUFAs) such as Omega-3 PUFAs are indispensable for the normal development, function, and maintenance of the central nervous system. Recently, Omega-3 PUFA and derivatives have been shown to modulate neuroinflammation and mitigate brain parenchymal damage after TBI by regulating NLRP3 inflammatory vesicle release, lymphatic pathways, and aquaporin 4 (AQP4), and by promoting secretion of the antioxidant glutathione (GSH) as well as neurotrophic factors to improve neurovascular function [23–25]. Our previous studies also demonstrated that Omega-3 PUFA can inhibit the transformation of microglia to a pro-inflammatory phenotype, thereby reducing the production and release of inflammatory factors [26, 27]. However, the mechanisms by which Omega-3 PUFA regulates microglia phenotype and differential exosome expression is not fully understood.

In this study, we examined the contributions of the ADAM17/TNF-α/NF-κB signaling axis to the regulation of microglia phenotype, exosome secretion profile, neurodegeneration, and neurological dysfunction in TBI model mice. We report that Omega-3 PUFA improves neurological function after TBI by inhibiting the ADAM17/TNF-α/NF-κB pathway in microglia and by promoting the secretion of exosomes containing nerve growth factor (NGF) and ensuing activation of the neuronal NGF/TrkA pathway. This study thus provides a theoretical basis for clinical Omega-3 PUFA supplementation to treat TBI.

Material And Methods

Animals

All animal experiments were approved by the 900th Hospital Ethics Committee (Fuzhou, China) and conducted under its strict supervision. Adult male C57BL/6 mice weighing 22–28g were purchased from the experimental animal facility of Fujian Medical University. During the experimental period, all animals were housed at room temperature (24–26°C) under a 10h/14h light/dark cycle with free access to standard rodent chow and water.

Experimental Animal Grouping
Mice were randomly divided into five groups (n = 18 mice per group): control, TBI, TBI + ω-3 PUFA, TBI + ω-3 PUFA + NGF inhibitor, and TBI + ω-3 PUFA + PMA (Adam17 activator). Nine mice per group received neurological assessment and the remaining mice were used for histological and molecular studies. A TBI mice model was established using a controlled cortical impact (CCI) device [23]. Briefly, mice were deeply anesthetized with 3% isoflurane in a Plexiglas container and maintained under anesthesia with 1.5% isoflurane using a small animal anesthesia machine (RWD Life Science Co., Shenzhen, China). The anesthetized mice were placed in a stereotaxic frame and the skull was exposed by cutting the scalp along the midline. A hole of approximately 3.0 mm in diameter was drilled midway between the right fontanelle and the lambda and 2.0 mm lateral to the midline. The exposed cortical surface was struck with a 2 mm metal flat-head impactor (Brain and Spinal Cord Impactor, 68099H, RWD Life Science) at 5 m/s to a depth of 3 mm and with a strike dwell time of 100 ms. After the strike, the scalp was sutured and the mouse placed on a heating pad until recovery from anesthesia. Control animals underwent the same surgical procedure but without cortical impact. Thirty minutes after TBI, the TBI + Omega-3 PUFA, TBI + Omega-3 PUFA + PMA, and TBI + Omega-3 PUFA + NGF inhibitor groups were given intraperitoneal injections of Omega-3 PUFA (3 mg/kg docosahexaenoic acid [DHA]), NGF inhibitor (Ro 08-2750, 13.75 mg/kg), and (or) ADAM17 activator (PMA, 100 µg/kg) as indicated, while the remaining groups were injected with the same volumes of 0.9% NaCl as a control.

**Bv2 Cell Culture And Lps Stimulation**

The mouse microglia cell line BV2 was obtained from the Chinese Cell Line Resource Infrastructure (Beijing, China) and cultured at 37°C in humidified air containing 5% CO₂. The medium was composed of 90% Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Frederick, MD, USA), 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), and 1% antibiotics (100 U/mL penicillin and 100 g/mL streptomycin). Cells were activated by treatment with LPS (100 ng/mL)

**Culture Of Primary Neurons**

Primary neurons were isolated from the cortex of sham, TBI, and TBI + Omega-3 PUFA group mice, seeded on 6-well plates coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) at approximately 7 × 10⁵ cells/well, and cultured in DMEM. Four hours after seeding, the medium was changed to Neurobasal medium (Gibco, Carlsbad, NM) supplemented with B-27 (Gibco). Cells were incubated in a humidified incubator at 37°C under a 5% CO₂ atmosphere. Cultures were used for experiments 7–10 days after seeding.

**Isolation, Identification, And Labeling Of Microglia-derived Exosomes**

BV2 cells were washed twice with PBS and grown in medium which removed any spontaneously released exosomes. The vehicle group was treated with PBS, the LPS group with LPS (200 ng/mL), and the LPS +
ω-3PUFA group with LPS (200 ng/mL) and DHA (200 ng/mL) for 5 h. After 48 h of cell culture, the supernatant was collected and serially ultracentrifuged at 4°C. Exosomes were isolated from supernatant using a whole exosome isolation kit (4478359, Invitrogen, USA).

Exosome concentration and size distribution were measured by Brownian motion using a NanoSight LM10 nanoparticle analyzer (Malvern) equipped with a Blue488 nm laser and high-sensitivity sCMOS camera at a constant temperature of 25°C. The syringe pump speed was set to 20 Units. The exosome suspension was allowed to settle for 90s before acquisition and then three measures were acquired for 30s. The three repeated measures were averaged to determine the particle size distribution and nanoparticle concentration. Exosomes were also labelled using a PKH26 kit, washed and diluted with PBS, and then examined by transmission electron microscopy (JEM-2100F, Japan) for exosome uptake experiments.

**Protein Mass Spectrometry**

The isolated and purified exosome samples were diluted 8-fold in 50 mM ammonium bicarbonate (Sigma) and digested overnight using mass spectrometry grade Trypsin/LysC (Promega) at a 1:10 (v/v) enzyme concentration. The next day, samples were acidified to pH < 4.0 using 1% TFA (Sigma) and the release peptide contents identified by either a Q-accurate HF (Thermo Scientific) or an orbital Lapp Lumos hybrid mass spectrometer, both coupled on-line to an EASY-nLC II 1200 chromatography system. Briefly, samples were loaded onto a 50 cm fused silica emitter with ReproSIL-Pur C18-oven. The peptides were eluted in Q-Exactive HF using a two-step gradient of 80% acetonitrile (solvent B) at a flow rate of 300 nL/min for 125 min, 2–20% 0.1% formic acid over 73 min, and 41% formic acid for 93 min. The peptides were injected into the mass spectrometer by electrospray using a nanoelectrospray ion source (Thermo Scientific), and an active background ion reduction device (ABIRD, ESI source solution) was used to reduce potential airborne contaminants. Data were acquired in positive ion mode using Data Dependent Acquisition (DDA) and Xcalibur software (Thermo Scientific). The mass range for the full scan was set to 375–1500 m/z with a resolution of 60,000 Units. The injection time was set to 20 ms and the target value was 3E6 ions. The MS files were processed by MaxQuant software version 1.6.1.0 for all experiments.

**Cell Survival Assay**

Cell viability was determined using Cell Counting Kit-8 solution (CCK-8, Dojindo, Kumamoto, Japan). The CCK-8 solution was added to each well for 2 hours and absorbance measured at 450 nm using a microplate reader as an estimate of viable cell number.

**Western Blotting**

Western Blotting
Proteins were extracted from tissue samples surrounding the cortical injury site using Radioimmunoprecipitation Assay (RIPA) Lysis Buffer (Santa Cruz Biotechnology, Dallas TX, USA). Briefly, lysates were incubating on ice and centrifuged to isolate soluble proteins in the supernatant. Protein concentrations in supernatant were determined using a BCA protein assay kit (Abcam, Cambridge, UK), then equal amounts per gel lane (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk for 2 h at room temperature and then incubated with primary antibodies targeting CD16+ (1:500; Santa Cruz), ADAM17 (1:500; Abcam), and TNF-α (1:500; Santa Cruz), TNFR1 (1:500; Santa Cruz), p-IκB (1:500; Santa Cruz), NF-κB p65 (1:500; Santa Cruz), p-NFκB p65 (1:500; Santa Cruz), caspase-3 (1:3,000; Abcam), Bax (1:1,000; Abcam), Bcl2 (1:2,000; Abcam), NGF (1:1000, Abcam), TrkA (1:1,000; Abcam), and p-TrkA (1:1,000; Abcam). After incubation with an appropriate secondary antibody, immunolabeling was visualized using the Millipore ECL Western Blotting Detection System (Millipore, Billerica, MA, USA). Target band density (gray level) was measured using UN-Scan-It 6.1 software (Silk Scientific Inc., Orem, UT, USA) and normalized to β-actin (1:2000, Abcam) or GAPDH (1:2000, Abcam) density as the gel loading control.

**Immunofluorescence**

Cultured cells treated as indicated were fixed in formaldehyde, embedded in paraffin, cut into 4-µm thick sections, dewaxed in xylene, rehydrated in gradient alcohol, then subjected to antigen repair, and incubated at 4°C with antibodies against CD16 (1:500, Abcam), NeuN (1:500; Abcam), and NF-κB p65 (1:500; Santa Cruz). After washing, sections were incubated with secondary antibodies for 1 h at room temperature. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Immunopositive cells in 5 selected areas were counted under a fluorescence microscope by an observer blinded to treatment history.

**Immunohistochemistry**

Brain tissue samples were fixed in formaldehyde, embedded in paraffin, cut into 4-µm thick sections, dewaxed in xylene, rehydrated in gradient alcohol, and incubated with an antibody against ionized calcium-binding adapter molecule (Iba)-1 (1:500; Abcam, Cambridge, UK). Stained sections were then washed, incubated with secondary antibody for 1 h at room temperature, and examined under fluorescence microscopy. Five sections from each animal were graded for fluorescent signal intensity as follows: 0, no positive signal; 1, very few positive cells; 2, moderate positive cells; 3, large number of positive cells; 4, maximum number of positive cells.

**Nissl Staining**
Tissue specimens encompassing the TBI area were fixed in formaldehyde, embedded in paraffin, cut into approximately 4-µm thick sections, dewaxed in xylene, rehydrated in gradient alcohol, and treated with Nissl staining solution for five minutes. The number of surviving neurons was counted in 5 randomly selected regions of interest (ROIs) by a researcher who was unaware of the experimental group.

**Tunel Staining**

Apoptotic cells were counted by TUNEL assay using the Apoptosis Cell Kit (Roche Inc., Indianapolis, IN, USA). Sections were washed in PBS and incubated with NeuN (1:500; Abcam) overnight at 4°C and then with the TUNEL reaction mixture for 1 h at 37°C. The number of TUNEL-positive neurons in the area surrounding the TBI site was counted under high-powered microscopy (×400) in five randomly selected ROIs.

**1.1 Enzyme-linked immunosorbent assays (ELISAs)**

Concentrations of TNF-α, IL-1β, IL-6, and IFN-γ were measured in brain tissue using ELISA kits (Jingmei Biotechnology, Jiangsu, China). Standards and samples were incubated with horseradish peroxidase (HRP) after binding to the respective antibodies according to the manufacturer's instructions. Optical density (OD) values were converted to concentrations according to standard curves.

**Blood–brain Barrier Permeability Measures By Evans Blue Staining**

Blood–brain barrier permeability was estimated by the extravasation of Evans Blue (EB) following cerebrovascular injection. Briefly, EB solution (Sigma-Aldrich; 2% in saline; 5mL/kg) was injected through the common carotid artery 3 days after TBI and 2 h prior to sacrifice [29]. After sacrifice, mice were perfused with refrigerated PBS (4 ± 1°C) via the heart until no blood flowed out, and then with PBS containing 4% paraformaldehyde. Brains were removed, weighed, and homogenized in 600 µL of 7.5% (w/v) trichloroacetic acid solution. Homogenates were centrifuged and absorption of the supernatant was measured by spectrophotometry at 620 nm. The amount of Evans blue was calculated from a standard curve and expressed as micrograms of per gram of brain tissue (wet weight).

**Analysis Of Cerebral Edema**

At 72 hours after TBI, brains were quickly removed and weighed on pre-weighed aluminum foil. The samples were then baked in an oven at 90°C for a further 72 h and re-weighed. Brain water content was calculated as (total wet brain weight - dry brain weight) / total wet brain weight × 100%.

**Assessment Of Motor And Neurobehavioral Function**
Mice were examined for motor (muscle status and abnormal movements), sensory (visual, tactile and balance sensations), and reflex responses using a modified neurological severity score (mNSS) [28]. Each test was scored 0 (normal) or 1 (impaired) for a maximum total score of 18. Performance was then gaged as normal (total score of 0), mild impairment (1–6), moderate impairment (7–12) or severe impairment (13–18).

The Garcia test of neurological function was also used to evaluate spontaneous movement, axial sensation, touch proprioception, limb symmetry, lateral transfer, forelimb extension, and climbing ability. Each test was scored from 0 (poor) to 3 (normal) for a maximum total score of 21, with higher scores indicating better overall performance. Both tests were conducted at different times by experimenters unaware of the grouping information.

### Statistical Analysis

All statistical analyses were performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± standard deviation. Two groups were compared by independent-samples t-test and more than two groups by one-way analysis of variance (ANOVA) with post hoc Student-Newman-Keuls tests for pair-wise comparisons. A p < 0.05 (two-tailed) was considered statistically significant for all tests.

### Results

**Omega-3 PUFA inhibits pro-inflammatory transformation of microglia in association with the ADAM17/TNF-α/NF-κB pathway**

Inflammation following TBI is initiated by the phenotypic transformation (or activation) of resident microglia in response to intracellular constituents released from death or dying cells (damage-associated molecular patterns or DAMPs) [22]. Activated microglia release the proinflammatory factor TNF-α, which is converted to an active soluble form by ADAM17. Therefore, we first examined if the neuroprotectant Omega-3 PUFA influences the phenotype of cultured BV2 microglial cells following treatment with the inflammation inducer LPS. Immunofluorescence analysis and western blotting (WB) revealed increased expression of the pro-inflammatory marker CD16 after LPS stimulation, a response that was significantly suppressed by ω-3 PUFA (p < 0.05). However, co-addition of the ADAM17 activator phorbol 12-myristate 13-acetate (PMA) reversed this anti-inflammatory effect of ω-3 PUFA (p < 0.05) (Fig. 1A–C). Treatment with ω-3 PUFA also reduced the expression levels of pro-inflammatory factors TNF-α, interleukin (IL)-β, IL-6, and interferon (IFN)-γ after LPS induction as measured by ELISA (p < 0.05), while PMA again abrogated this anti-inflammatory effect (Fig. 1D).

To investigate the mechanism by which Omega-3 PUFA regulates the LPS-induced phenotypic transformation of microglia, we examined ADAM17 and TNF-α/NF-κB pathway proteins by WB.
Treatment of BV2 cells with LPS alone significantly increased the expression levels of ADAM17, TNF-α, and type 1 TNF receptor (TNFR1) as well as the phosphorylation levels of NF-κB inhibitory protein (p-IκB) and NF-κB (Fig. 1E-F), indicating enhanced NF-κB nuclear translocation and transcriptional activity (ref). Cotreatment with ω-3 PUFA reduced ADAM17 and TNF-α/NF-κB-related pathway protein expression (all p < 0.05 compared to LPS alone). However, addition of PMA reversed the (anti-inflammatory) effects of ω-3 PUFA on ADAM17 expression and TNF-α/NF-κB pathway activity (Fig. 1E-F). These findings strongly suggest that the inhibitory effect of ω-3 PUFA on pro-inflammatory microglia is related to suppression of the ADAM17/TNF-α/NF-κB signaling axis.

Omega-3 Pufa Increases Ngf Levels In Microglial Exosomes

Microglia are known to secrete exosomes with distinct content profiles depending on activation state. We speculated that Omega-3 PUFA could shift these contents toward an anti-inflammatory profile and examined this possibility by TEM and proteomics analysis of exosomes derived from BV2 cultures following the same treatment combinations as in Fig. 1. TEM of sectioned BV2 cells revealed a large number of disc-shaped vesicles in the cell periphery (Fig. 2A). After isolation from the extracellular medium, these disc-shaped vesicles ranged from 55–120 nm in diameter (Fig. 2B) and highly expressed exosome markers such as CD9, TSG101, CD81, and CD63 (Fig. 2C). Mass spectrometry revealed that 129 proteins were specifically expressed in exosomes from microglia treated with LPS + Omega-3 PUFA, while 224 proteins were co-expressed in LPS and LPS + Omega-3 PUFA treatment groups, 59 proteins were co-expressed in Vehicle and LPS + Omega-3 PUFA treatment groups, and 36 proteins were present in exosomes from all three treatment groups (Fig. 2D). To understand the functional implications of this differential expression, we constructed heat maps and performed KEGG pathway enrichment analysis. The heatmap showed that the neuroprotective factor NGF was significantly enriched in exosomes from the LPS + Omega-3PUFA group. Further, KEGG analysis suggested that the set present only in exosomes from the LPS + Omega-3PUFA group was significantly enriched in proteins related to ‘GABAergic synapse’, ‘neuroactive ligand-receptor interaction’ and ‘other pathways’ (Fig. 2E, F). These results suggest that Omega-3 PUFA may protect neurons against inflammatory damage by promoting the release of exosomes containing neuroprotective signaling factors including NGF.

Omega-3 Pufa Inhibits Neuronal Apoptosis By Regulating Ngf Levels In Exosomes

Nerve growth factor contributes to neurogenesis as well as neuronal and synaptic repair after injury. Indeed, NGF is highly expressed and promotes neuronal activity after TBI [30]. To investigate the neuroprotective potential of NGF-enriched exosomes secreted by Omega-3 PUFA-stimulated microglia, we first tested the capacity of isolated exosomes to fuse with the membranes of mouse primary cortical neurons and deliver contents intracellularly, and then examined the effects of different exosome populations on neuronal survival. Confocal imaging showed that PKH-26-labelled exosomes (green) could be taken up by neurons (red) in vitro (Fig. 3A). Exosomes isolated from LPS-treated BV2 microglial
cultures reduced the survival rate of primary neurons and significantly increased the TUNEL-positive (apoptosis) rate ($p < 0.05$), while subsequent addition of NGF-rich exosomes from BV2 cells treated with LPS + Omega-3 PUFA significantly improved neuronal survival ($p < 0.05$). This protective effect was largely attenuated ($p < 0.05$) by NGF inhibitors (Fig. 3B–D). In accord with cell viability and TUNEL staining results, treatment with NGF-enriched exosomes inhibited the elevations in pro-apoptotic cleaved caspase-3 and Bax expression and the reduction in anti-apoptotic Bcl-2 expression induced by exosomes from LPS-treated microglia ($p < 0.05$) (Fig. 3E, F). Again, these anti-apoptotic effects were reversed by inclusion of an NGF inhibitor. Moreover, the expression levels of cleaved caspase-3, Bax, and Bcl-2 in neurons treated with exosomes of the LPS + Omega-3 PUFA + NGF inhibitor group did not differ from neurons treated with exosomes from the LPS-exposed group.

**Omega-3 Pufa Inhibits Microglia Conversion To A Pro-inflammatory Phenotype After Tbi**

Experimental TBI increased local expression of the activated microglia marker Iba-1, a response significantly reduced by post-TBI Omega-3 PUFA injection ($p < 0.05$). Consistent with culture findings, co-injection of the ADAM17 activator PMA reversed this effect on Iba-1 expression ($p < 0.05$) (Fig. 4A-B). Also in accord with findings in vitro, post-TBI Omega-3 PUFA injection reduced TNF-α, IL-1β, IL-6, and IFN-γ levels in the damaged area as measured by ELISA ($p < 0.05$), and this anti-inflammatory effect was also reversed by PMA co-injection (Fig. 4D).

In previous in vitro experiments, we found that Omega-3 PUFA inhibited microglial transformation to a pro-inflammatory phenotype and that this effect was associated with the ADAM17/TNF-α/NF-κB pathway suppression. Therefore, we investigated contributions of the ADAM17/TNF-α/NF-κB signaling axis to regulation of microglia phenotype by Omega-3 PUFA in TBI model mice. Western blotting results showed that the expression levels of ADAM17, TNF-α, and TNFR1 were increased in the damaged area, and that IκB and p-NFκB were also significantly upregulated ($p < 0.05$), while post-TBI Omega-3 PUFA injection inhibited the expression of ADAM17, TNF-α, and other pathway proteins ($p < 0.05$). However, co-injection of the ADAM17 activator PMA reversed the effects of Omega-3 PUFA as expression of TNF-α/NF-κB-related pathway proteins increased in parallel with ADAM17 expression (Fig. 4C, E, F). Also, immunofluorescence staining of sections from the injured site indicated that Omega-3 PUFA inhibited NF-κB nuclear translocation, and that this effect was attenuated by ADAM17 activator co-injection ($p < 0.05$) (Fig. 4G). In accord with cell culture findings, these TBI model results indicate that Omega-3 PUFA inhibits microglial activation to the pro-inflammatory phenotype following TBI by suppressing the ADAM17/ TNF-α/NF-κB pathway.

**Omega-3 Pufa Activates The Ngf/trka Pathway To Inhibit Neuronal Apoptosis After Tbi**
In previous experiments, we found that Omega-3 PUFA promoted the secretion of NGF-enriched exosomes from microglia, while our current in vitro studies suggest that this secretion can protect primary mouse cortical neurons against apoptotic death. To examine if this exosomal signaling mechanism also operates at the site of TBI, we examined the effects of Omega-3 PUFA on NGF pathway protein expression and activity within the damaged area. Western blotting showed that NGF and P75 neurotrophic factor receptor (P75NTR) were upregulated after TBI, while tyrosine protein kinase A phosphorylation (p-TrkA) was reduced (p < 0.05). NGF expression was further increased by post-TBI injection of Omega-3 PUFA. Similarly, p-TrkA expression was significantly increased (p < 0.05), but P75NTR expression was downregulated (Fig. 5A, B). This activation of the NGF/TrkA pathway was not markedly influenced by the ADAM17 activator PMA but was largely reversed by inhibition of NGF signaling (p < 0.05) (Fig. 5A, B). The Omega-3 PUFA-induced increase in NGF/TrkA pathway activity was associated with reduced TUNEL positivity in damaged areas (p < 0.05), and this reduction was greatly attenuated by co-administration of the NGF inhibitor (Fig. 4C, D), indicating that neuroprotection was mediated primarily by enhanced NGF signaling, potentially via microglial exosomes. Consistent with TUNEL staining results, post-TBI Omega-3 PUFA injection reduced cleaved caspase-3 and Bax expression and increased Bcl-2 expression at the injury site compared to the TBI group (p < 0.05). Also in accord with TUNEL staining experiments, this inhibitory effect on neuronal apoptosis was reversed by suppression of the NGF/TrkA pathway (p < 0.05) (Fig. 4E, F).

**Omega-3 Pufa Suppresses Secondary Degeneration And Improves Neurological Function After Tbi**

Nissl staining revealed that post-TBI Omega-3 PUFA injection significantly reduced the shrinkage of neurons and the appearance of vacuoles in the area of injury (p < 0.05). These effects were weakened by co-administration of the ADAM17 activator PMA and more markedly suppressed by the NGF inhibitor (p < 0.05) (Fig. 5A, B). In addition, neurological function was severely impaired after TBI as assessed by mNSS and Garcia scores, while neurological function scores were significantly improved (lower mNSS and higher Garcia scores) by Omega-3 PUFA on day 3 post-TBI. Consistent with histopathological results, this function improvement was reduced by the NGF inhibitor (p < 0.05) and also by PMA (Fig. 5C, D), consistent with Western Blot experiments showing that Omega-3 PUFA suppressed pro-inflammatory and apoptotic signaling, while PMA and the NGF inhibitor mitigated these effects. Collectively, these results suggest that Omega-3 PUFA improves neurological function following TBI by suppressing transition of local microglia to the pro-inflammatory phenotype, a state in which these cells release exosomal factors inducing the ADAM17/ TNF-α/NF-κB pathway in neurons, and by maintaining the microglial phenotype that releases exosomes containing neuroprotective factors such as NGF, leading to activation of the neuronal NGF/TrkA pathway.

The level of brain edema following TBI is an important prognostic indicator, and edema severity (brain water content) correlates with neuroinflammation [31]. Consistent with functional assessments, brain water weight was significantly reduced by Omega-3 PUFA treatment, an effect partially reversed by PMA.
(p < 0.05) (Fig. 5E). However, unlike neurological function scores, the effect of ω-3 PUFA on brain water content was reversed by the ADAM17 activator PMA (p < 0.05). Results of Evans Blue (EB) assay for BBB integrity were in accord with the changes in brain water weight, with the rise in dye permeability following TBI suppressed by Omega-3 PUFA, and this protective effect on the BBB reversed by PMA and NGF inhibition (Fig. 5F, G).

Discussion

Traumatic brain injury is one of the most frequent conditions treated by neurologists, neurosurgeons, and rehabilitation staff, and prognosis for full functional recovery is generally poor, placing a heavy burden on families, healthcare systems, and social services [32]. After TBI, microglia are transformed to a pro-inflammatory phenotype that promotes further neuronal damage (secondary injury) and also damages the neurovascular elements comprising the BBB [33]. Therefore, therapeutic strategies to suppress microglia-induced neuroinflammation after TBI may reduce secondary neural damage, prevent progressive neurodegeneration, and facilitate functional preservation or improvement. In this study, we demonstrate that Omega-3 PUFA can prevent microglial activation and suppress the neuroinflammatory ADAM17/TNF-α/NF-κB pathway. In addition, we found that Omega-3 PUFA promotes the microglial secretion of exosomes enriched in NGF (and possible other neuroprotective factors) that are absorbed by local neurons and activate the NGF/TrkA pathway, resulting in reduced neuronal apoptosis and neurological improvement.

Omega-3 PUFAs are essential components of mammalian cell membranes and participate in a variety of processes such as development and tissue damage repair. However, many mammals are unable to synthesize Omega-3 PUFAs, so these must be obtained through the diet (and/or pharmaceutical supplements in the case of humans). Several studies have shown that Omega-3 PUFAs can exert anti-inflammatory and anti-oxidant effects after TBI and improve neuronal function [34]. Nuclear factor-κB is a transcription factor that regulates the phenotype of microglia by controlling the expression of cytokines, immunoreceptors, antigen-presenting proteins, and proteins involved in chemotaxis and morphogenesis [35]. Binding of TNF-α to its receptor TNFR1 can activate IKK-α/β to induce IκB phosphorylation, which in turn promotes NF-κB nuclear translocation to regulate genes determining microglial phenotype [13, 14]. However, TNF-α levels are regulated by ADAM17 on the surface of microglia [36, 37]. Therefore, we hypothesized that Omega-3 PUFA may inhibit the TNF-α/NF-κB pathway by reducing ADAM17 activity and concomitant TNF-α release from microglia. Consistent with this notion, Omega-3 PUFA-induced downregulation of the microglial pro-inflammatory phenotype markers CD16 and Iba-1 as well as suppression of ADAM17/TNF-α/ NF-κB pathway protein expression levels were abrogated by an ADAM17 activator. Moreover, Omega-3 PUFA-induced suppression of neuroinflammation, maintenance of BBB integrity, neuroprotection, and preservation of neurological function were variably reversed by this same ADAM17 activator (PMA).

Previous studies have shown that the anti-apoptotic and protective effects of Omega-3 PUFA on damaged neurons are associated with microglia activity [24, 38, 39] but the precise mechanisms were
Exosomes are cell membrane-derived microvesicles that mediate intercellular communication through the plasma membrane fusion and intracellular release of contents such as growth factors, microRNAs, bioactive lipids, and various nutrients [15, 40]. Several studies have shown that some microglial exosomes can improve neuronal function after TBI [41, 42] by transferring various RNAs (e.g., miRNAs, IncRNAs, and cirRNAs). In a previous study, we demonstrated that Omega-3 PUFA can inhibit microglial conversion to a pro-inflammatory phenotype via the ADAM17/TNF-α/NF-κB pathway and that the contents of microglia exosomes differed according to phenotype. However, unlike these previous studies, we examined the small protein profile and found that microglial exosomes contained Intercellular adhesion molecule-1 (Icam-1), heat shock protein 8 (Hspa8), GTPase homolog1 (GUF-1), and fibrinogen gamma (FGG) after Omega-3PUFA treatment, and KEGG analysis showed that contents were enriched in proteins associated with ‘GABAergic synapse’, ‘neuroactive ligand-receptor interaction’, and ‘TGF-beta signaling pathway’. Among these differentially enriched proteins, the most intriguing was NGF, a neurotrophic factor that can promote neuronal survival and development by binding with the cell surface receptor TrkA at growth cones and synapses, followed by retrograde transport of the complex to the nucleus [43]. Moreover, the anti-inflammatory and anti-apoptotic effects of these exosomes in vitro were suppressed by a NGF antagonist, suggesting that NGF is a major contributor to the overall neuroprotective efficacy of Omega-3 PUFA. Further, NGF levels and NGF/P75NTR pathway activity were mildly upregulated in the damaged area of mouse cortex following experimental TBI, and NGF was further increased by Omega-3 PUFA. Notably, however, Omega-3 PUFA suppressed the pro-apoptotic NGF/P75NTR pathway and activated the neuroprotective NGF/TrkA pathway. The TrkA receptor is phosphorylated upon binding to NGF and activates several downstream signaling pathways such as PLC-γ1, MAPK, and PI3K to promote neuronal growth and survival [43]. This change in NGF pathway activity in the presence of Omega-3 PUFA is consistent with results from Nissl staining showing reduced neuronal damage in TBI model mice receiving Omega-3 PUFA. We suggest that these improvements may result from both activation of NGF/TtkA pathway signaling and suppression of NGF/P75NTR pathway signaling.

This study has several limitations. First, we did not specifically investigate whether Omega-3 PUFA acts directly on the ADAM17 protein or identify the specific site of action. Further, the molecular mechanisms by which Omega-3 PUFA regulates exosomal NGF levels are still unclear. In future studies, we will further explore the mechanisms underlying Omega-3 PUFA modulation of microglia phenotype and changes in exosomal NGF to identify additional targets for potential therapeutic manipulation.

**Conclusions**

Omega-3 PUFA can suppress ADAM17 activity, thereby inhibiting the release of TNF-α by microglia. In addition to blocking the pro-inflammatory activity of microglia, Omega-3 PUFA can promote the release of exosomal nerve growth factor from microglia and activate the neuroprotective TNF-α/NF-κB pathway. In concert, these two effects protect neurons against apoptosis and preserve neurological function following traumatic brain injury.
Abbreviations

TBI traumaic brain injury
TNF-α Tumor necrosis factor-α
ADAM17 extracellular A disintegrin metalloproteinase 17
LPS lipopolysaccharide
IL interleukin
NF-κB nuclear factor-κB
NGF nerve growth factor
CCI controlled cortical impact
DHA docosahexaenoic acid
PMA phorbol 12-myristate 13-acetate
TNFR1 type 1 TNF receptor
p-IκB phosphorylated NF-κB inhibitory protein
TEM transmission electron microscopy
P75NTR P75 neurotrophic factor receptor
p-TrkA phosphorylated tyrosine protein kinase A
EB Evans Blue
Icam-1 intercellular adhesion molecule-1
Hspa8 heat shock protein 8
GUF-1 GTPase homolog1
FGG fibrinogen gamma
BBB blood-brain barrier

Declarations

Authers’ Contributions:
Long Lin: Conceptualization (equal); Formal analysis (equal); Methodology (equal); Writing-original draft (equal). Shaorui Zheng: Formal analysis (equal); Methodology (equal); Software (equal). Jinqing Lai: Data curation (equal); Methodology (equal). Dan Ye: Project administration (equal); Formal analysis (equal). Qiaomei Huang: Project administration (equal); Formal analysis (equal). Zhe Wu: Methodology (equal); Formal analysis (equal). Xiangrong Chen: Formal analysis (equal); Methodology (equal); Writing-review & editing (equal); Funding acquisition (equal). Shousen Wang: Conceptualization (equal); Funding acquisition (equal); Project administration (equal); Supervision (equal); Writing-review & editing (equal).

Data Availability

The datasets generated and/or analyzed for the present study are available from the corresponding author on reasonable request.

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Conflicts of Interest:

The authors declare no competing financial interests.

References


Figures
Figure 1

Omega-3 PUFA inhibits the ADAM17/TNF-α/NF-κB pathway responsible for the transformation of BV2 microglia to the pro-inflammatory phenotype. (a) Immunofluorescence staining of lipopolysaccharide (LPS)-treated BV2 cultures showing that subsequent ω-3 PUFA treatment inhibits expression of CD16, a marker of the pro-inflammatory phenotype, and that cotreatment with the ADAM17 activator PMA abrogates the anti-inflammatory effect of ω-3 PUFA. (b, c) Western Blot analysis of BV2 cell lysates confirms that ω-3 PUFA reduces CD16 expression, while PMA co-treatment reverses this effect. (d) LPS
promotes the production of secreted inflammatory factors (TNF-α, IL-β, IL-6, and INF-γ) by (CD6+) BV2 cells, while ω-3 PUFA suppresses these responses and the effects of ω-3 PUFA are partially reversed by PMA. (e, f) Western blotting with phospho-antibodies demonstrates that ω-3 PUFA inhibits the ADAM17/TNF-α/NF-κB pathway in activated BV2 microglia while upregulation of ADAM17 by PMA reverses these effects. Values are expressed as the mean ± standard deviation (n = 6 independently treated cultures per group), *p < 0.05, **p < 0.01. Scale bars = 50 μm.

Figure 2

Omega-3 PUFA increases NGF levels in microglia exosomes. (a) Transmission electron micrographs showing a large number of vesicles around the periphery of BV2 cells in Vehicle, LPS, and LPD+ Omega-3 PUFA treatment groups. (b) Nanoparticle tracking analysis shows that these vesicles are distributed between 55–120 nm. (c) Western blot assays indicating that these vesicles express the exosomal markers CD9, Tsg101, and CD63. (d) Venn diagram showing differences in protein contents among microglial exosomes from Vehicle, LPS, and LPS+ Omega-3PUFA treatment groups. (e) Heat map illustrating that small molecule proteins are expressed at different levels in the Vehicle, LPS, and LPS+ Omega-3PUFA treatment groups. Red indicates strong expression and blue weak expression. (f) KEGG pathway enrichment analysis showing that these small molecule proteins differ in molecular function between LPS and LPS+ Omega-3PUFA groups. Red indicates strong enrichment and blue weak.
Figure 3

Omega-3 PUFA inhibits neuronal apoptosis induced by microglial exosomes by upregulating exosomal NGF. (a) Confocal image showing PKH-26-labelled exosomes (green) being taken up by neurons (red) in vitro. (b) Neurons were treated with different groupings of BV2 cell exosomes. The number of surviving
neurons was determined by cell counting assay. (c, d) Neurons were treated with different groupings of BV2 cell exosomes and TUNEL-positive neurons were counted. (e, f) Western blots showing changes in cleaved caspase-3, Bax, and Bcl-2 protein expression in sham, LPS, LPS+ Omega-3 PUFA, and LPS+ Omega-3 PUFA+NGF inhibitor groups. Values are expressed as the mean ± standard deviation (n = 6 independently treated cultures per group), *p < 0.05, **p < 0.01. Scale bars = 50 μm.
Omega-3 PUFA inhibits microglia conversion to the pro-inflammatory phenotype in a mouse model of TBI. (a, b) Immunohistochemical staining experiments showing increased expression of the activated microglia marker Iba-1 at the site of TBI. Injection of Omega-3 PUFA reduces Iba-1 expression, an effect inhibited by cotreatment with PMA. (d) ELISA assays measuring the expression levels of inflammatory factors in sham, TBI, TBI+ Omega-3 PUFA, and TBI+ Omega-3 PUFA+PMA group mice. (c, e, f) Western blot analysis showing that Omega-3 PUFA injection inhibits activation of the ADAM17/TNF-α/NF-κB pathway and reduces pathway protein expression levels at the site of TBI. These effects are also reversed by PMA. (g) Immunofluorescence staining showing increased NF-κB p65 translocation to the neuronal nucleus at the TBI site. Injection of Omega-3 PUFA group blocks translocation, while cotreatment with PMA abrogates the effect of Omega-3 PUFA. Values are expressed as the mean ± standard deviation (n = 6 mice per treatment group), *p < 0.05, **p < 0.01. Scale bars = 50 μm.
Figure 5

Omega-3 PUFA activates the NGF/TrkA pathway to inhibit neuronal apoptosis after TBI. (a, b) Western blotting analysis showing increased NGF/P75NTR pathway protein expression and decreased TrkA phosphorylation in the TBI group compared to the Vehicle control group. Treatment with Omega-3 PUFA enhances NGF/TrkA pathway protein expression and reduces P75NTR expression, effects partially reversed by cotreatment with the ADAM17 activator (PMA) and more substantially reversed by an NGF
inhibitor. (c, d) TUNEL-positive rates in sham, TBI, TBI+ Omega-3 PUFA, TBI+ Omega-3 PUFA+PMA, and TBI+ Omega-3 PUFA+NFG inhibitor groups. (e, f) Omega-3 PUFA reduces cleaved caspase-3 and Bax expression levels and promotes Bcl-2 expression after TBI. Both PMA and NGF inhibitors abrogate Omega-3 PUFA actions, with the NGF inhibitor exerting the greater effect. Values are expressed as the mean ± standard deviation (n = 6 mice per treatment group), *p < 0.05, **p < 0.01. Scale bars = 50 μm.

Figure 6
Omega-3 PUFA improves neurological function after TBI (a, b) The proportion (%) of damaged neurons was significantly reduced by ω-3 PUFA, and this neuroprotective effect was reversed by PMA and the NGF inhibitor. Representative photomicrographs of Nissl-stained neurons are shown. (c, d) At 3 days post-TBI, mice receiving daily Omega-3 PUFA showed significant improvements in neurological function, which again were abrogated by PMA and the NGF inhibitor, with the NGF inhibitor having the more pronounced effect. (e) Omega-3 PUFA reduced brain water content 72 h after TBI, and brain water content was again increased by PMA and NGF inhibitor cotreatment. (f) Omega-3 PUFA reduces EB extravasation after TBI. Compared to the TBI+ Omega-3 PUFA group, the TBI+ Omega-3 PUFA+PMA and TBI+ Omega-3 PUFA+NFG inhibitor groups showed greater EB leakage at 72 h post-TBI. Values are expressed as the mean ± standard deviation (n = 6 mice per treatment group), *p < 0.05, **p < 0.01. Scale bars = 50 μm.

Figure 7

Omega-3 PUFA inhibits the transformation of microglia to the pro-inflammatory phenotype through suppression of the ADAM17/TNF-α/NF-κB pathway, and promotes secretion of NGF-enriched exosomes from microglia to protect neurons and reduce inflammation after traumatic brain injury.