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A post-stroke therapeutic regimen with omega-3 polyunsaturated fatty acids that promotes white matter integrity and beneficial microglial responses after cerebral ischemia

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Abstract

White matter injury induced by ischemic stroke elicits sensorimotor impairments, which can be further deteriorated by persistent proinflammatory responses. We previously reported that delayed and repeated treatments with omega-3 polyunsaturated fatty acids (n-3 PUFAs) improve spatial cognitive functions and hippocampal integrity after ischemic stroke. In the present study, we report a post-stroke n-3 PUFA therapeutic regimen that not only confers protection against neuronal loss in the gray matter but also promotes white matter integrity. Beginning 2 hours after 60 minutes of middle cerebral artery occlusion (MCAO), mice were randomly assigned to receive intraperitoneal docosahexaenoic acid (DHA) injections (10 mg/kg, daily for 14 days), alone or in combination with dietary fish oil (FO) supplements starting 5 days after MCAO. Sensorimotor functions, gray and white matter injury, and microglial responses were examined up to 28 days after MCAO. Our results showed that DHA and FO combined treatment facilitated long-term sensorimotor recovery and demonstrated greater beneficial effect than DHA injections alone. Mechanistically, n-3 PUFAs not only offered direct protection on white matter components, such as oligodendrocytes, but also potentiated microglial M2 polarization, which may be important for white matter repair. Notably, the improved white matter integrity and increased M2 microglia were strongly linked to the

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Author contributions

Y.S., X.H., Y.G. and J.C. designed the research. X.J., H.P., Z.W. and W.Z. performed the research. X.J. and Y.S. analyzed the data. X.J., H.P., D.H., J.C. and Y.S. wrote the manuscript. All authors reviewed and edited the manuscript.

Compliance with Ethical Standards

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Conflict of Interest

The authors declare that they have no conflict of interest.

mitigation of sensorimotor deficits after stroke upon n-3 PUFA treatments. Together, our results suggest that post-stroke DHA injections in combination with FO dietary supplement benefit white matter restoration and microglial responses, thereby dictating long-term functional improvements.

Keywords

myelin; oligodendrogenesis; corpus callosum; microglial polarization

Introduction

White matter, consisting of axonal fiber bundles, myelin-ensheathed axons and myelin-producing oligodendrocytes, plays a fundamental role in transmitting nerve signals and coordinating communication between brain regions [1, 2]. In human stroke, white matter occupies about half of the lesion volume and is an important cause of long-term sensorimotor deficits and cognitive decline [3–5]. Many neuroprotective drugs that showed promise in preclinical testing failed in clinical stroke trials [6]. One major concern is that most, if not all, preclinical studies focus on the protection of gray matter. Therefore, strategies that battle both gray and white matter injury and/or boost white matter repair are urgently needed for the clinical translation of successful preclinical stroke therapies.

White matter injury is characterized by demyelination and loss of axonal integrity [2, 7]. Demyelination, or destruction of the myelin sheath, if unrepaired, causes degradation of the naked axons, eventually leading to irreversible neurological disability [1]. White matter repair, including axonal regeneration, oligodendrogenesis, and the myelination of demyelinated or newly generated axons, rebuilds the neuronal circuits and restores axonal conduction [7–11]. Both white matter injury and repair are remarkably influenced by the functional status of the surrounding glial cells, such as astrocytes and microglia [12, 13]. For example, activated microglia can exert dualistic impacts on the white matter in a phenotype-dependent manner. Proinflammatory microglial responses are generally considered to exacerbate oligodendrocyte cell death and demyelination [14, 15], whereas the alternatively activated microglia (the so called “M2” microglia) can resolve local inflammation and promote remyelination, thereby facilitating white matter repair [16, 17]. To this end, therapeutic interventions that are capable of enhancing white matter restoration, either directly through actions on oligodendrocytes, or indirectly through modulation of microglial responses, are promising in improving the functional outcomes after stroke.

Long-term prophylactic dietary supplementation of omega-3 polyunsaturated fatty acids (n-3 PUFAs) offers potent protection against ischemic brain injury [18–20]. Furthermore, acute treatment after the onset of stroke with n-3 PUFAs, *e.g.* docosahexaenoic acids (DHA), appears to be effective in ameliorating neurological deficits and reducing neuronal loss up to 7 days after cerebral ischemia [21–24]. We recently observed that repeated administration of n-3 PUFAs, beginning at 2 h after post-ischemic reperfusion, improved spatial learning and memory in a mouse stroke model, at least in part through enhancing the hippocampal integrity [25]. However, it remains unknown whether a post-stroke n-3 PUFA treatment

regimen can promote white matter restoration and improve long-term sensorimotor function recovery.

It has been reported that n-3 PUFAs directly protect oligodendrocytes against excitatory cell death [26]. Moreover, we found that n-3 PUFAs potently induce M2 polarization in cultured microglia [27]. Both of these actions by n-3 PUFAs could contribute to white matter protection and/or restoration after stroke. Therefore, the present study was designed to determine the efficacy of post-stroke administration of n-3 PUFAs on promoting white matter integrity and sensorimotor functions using a mouse model of transient focal cerebral ischemia (tFCI). We report here a post-stroke n-3 PUFA treatment regimen that improves white matter restoration by promoting both oligodendrocyte survival and beneficial microglial responses.

Materials and methods

Animals

C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Animals were housed in a temperature- and humidity-controlled facility with a 12-h light-dark cycle. Food and water were available *ad libitum*. Efforts were made to minimize animal suffering and the number of animals used.

Transient focal cerebral ischemia model

tFCI was induced in adult male C57BL/6J mice (10–12 weeks old) by intraluminal occlusion of the left middle cerebral artery (MCA) for 1 h [28]. Experimental procedures were performed following the *Stroke Therapy Academic Industry Roundtable (STAIR)* guidelines. Briefly, mice were anesthetized with 3% isoflurane vaporized in 67%:30% N₂O/O₂ until they were unresponsive to the tail pinch test. Mice were then fitted with a nose cone blowing 1.5% isoflurane for anesthesia maintenance. A 7-0 suture with a silicon-coated tip was introduced into the common carotid artery, advanced to the origin of the MCA, and left undisturbed for 1 h. Rectal temperature was maintained at $37.0 \pm 0.5^{\circ}\text{C}$ during surgery with a temperature-controlled heating pad. To confirm the success of MCA occlusion and reperfusion, regional cerebral blood flow (rCBF) was measured using laser-Doppler flowmetry before, during, and after MCA occlusion (MCAO). Animals that did not show a CBF reduction of at least 75% of baseline levels or died immediately after ischemia induction or reperfusion (less than 10%) were excluded from further experimentation.

Delayed n-3 PUFA treatment after stroke

Immediately after the MCAO surgery, mice were randomly assigned to 3 groups with the use of a lottery-drawing box: 1) Vehicle control group. Mice were fed a regular laboratory rodent diet (Prolab Isopro RMH 3000 5P76; LabDiet, St. Louis, MO, USA) which has an inherently low n-3 PUFA concentration (0.36%), and received injections of 0.9% NaCl (300 μl per day, *i.p.* 2 h after MCAO, and then daily for 14 days). 2) DHA injection group. Mice were fed a regular diet, and received injections of DHA (10 mg/kg body weight, diluted with 300 μl of 0.9% NaCl, *i.p.* 2 h after MCAO, and then daily for 14 days). This dose of DHA injections was determined in a pilot study, which showed the therapeutic window of 2.5–10

mg/kg in the MCAO/reperfusion model (data not shown). 3) Combined DHA injection and fish oil dietary supplementation group. Mice were fed a diet supplemented with n-3 PUFAs (DHA and EPA, triple strength n-3 fish oil, Puritan's Pride, Oakdale, NY, USA; final n-3 PUFA concentration 4%) [18] 5 days after MCAO for up to 28 days, and received injections of DHA (10 mg/kg body weight, diluted with 300 μ l of 0.9% NaCl, *i.p.* 2 h after MCAO, and then daily for 14 days). We had quantified the food intakes by mice before and after MCAO (60 min)/reperfusion and found that their food intakes decrease after stroke, but fully recover at 4–5 days. Therefore, we started fish oil supplementations at 5 days after stroke to ensure all mice received approximately equal amount of n-3 PUFA supplementation every day. All outcome assessments were performed by investigators blinded to experimental group assignments.

Neurobehavioral tests

Before and after MCAO, sensorimotor functions of the mice were assessed by the cylinder and rotarod tests. The asymmetry of forelimb use was evaluated by the cylinder test as we described previously [28] before and at 3, 5, 7, 9, 11, 13, 15, 19, 23, 28 days after MCAO. Briefly, mice were placed in a transparent cylinder (9 cm in diameter and 15 cm in height) for 10 min. A camera was fixed above the cylinder to record all the forelimb movements of the mice. Videotapes were analyzed in slow motion, and forepaw (left/right/both) use during the first contact against the cylinder wall after rearing and during lateral exploration was recorded. Preference of the non-impaired forepaw (left) was calculated as a relative proportion of right forepaw contacts: $(\text{left-right})/(\text{left+right+both}) \times 100\%$ (asymmetric rate). Uninjured mice typically show no preference for either forepaw, whereas injured mice have increased left forepaw preference depending on the severity of the injury. The rotarod test was performed before and at 3, 5, 7, 10 days after MCAO to assess motor functions [28]. Briefly, mice were forced to run on a machine with accelerated rotating drums (IITC Life Science Inc., Woodland Hills, CA, USA). The time at which mice fell off the rod was recorded (latency to fall). The rotating speed of the rod was set to start at 4 rpm and accelerate to 40 rpm in 300 seconds. Mice were trained for 3 trials per day from 3 days before the surgery. The average time of the 3 trials during the last day of training was recorded as pre-surgery baseline value. After surgery, 5 trials were performed on each testing day with intervals of 5 min between each trial, and the data for trials #3–5 were used to calculate the mean latency to fall on that day.

Immunohistochemistry and image analysis

At 14 or 28 days after MCAO, mice were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Mouse brains were harvested and cryoprotected in 30% sucrose in PBS. Frozen serial coronal brain sections (25 μ m thick) were prepared on a cryostat (Microm HM459, Thermo Scientific). Brain sections were blocked with 5% donkey serum in PBS for 1 h, followed by incubation with primary antibodies for 1 h at room temperature and overnight at 4°C. After a series of washing, sections were incubated with donkey secondary antibodies conjugated to Alexa Fluor 488 or Cy3 (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Alternate sections from each experimental condition were incubated in all solutions except the primary antibodies to assess non-specific staining. Sections were mounted and

coverslipped with Fluoromount-G containing 4', 6-diamidino-2-phenylindole (DAPI; Southern Biotech, Birmingham, AL, USA). The following primary antibodies were used: rabbit anti-NeuN (1:500; EMD Millipore, Billerica, MA, USA), rabbit anti-myelin basic protein (MBP; 1:500; Abcam, Cambridge, MA, USA), mouse anti-nonphosphorylated neurofilaments (SMI-32; 1:1000; Abcam), mouse anti-adenomatous polyposis coli (APC; 1:400; EMD Millipore), rabbit anti-microtubule-associated protein 2 (MAP2; 1:200; Santa Cruz Biotechnology, Dallas, TX, USA), rat anti-CD16/32 (1:500; BD Biosciences, San Jose, CA, USA), goat anti-CD206 (1:500; R&D Systems, Minneapolis, MN, USA), rabbit anti-Iba1 (1:1000; Wako, Richmond, VA, USA). Images were acquired using an inverted Nikon Diaphot-300 fluorescence microscope equipped with a SPOT RT slider camera and Meta Series Software 5.0 (Molecular Devices, Sunnyvale, CA, USA). Alternatively, images were captured with an Olympus Fluoview FV1000 confocal microscope using FV10-ASW 2.0 software (Olympus America, Center Valley, PA, USA).

Analysis of the images was performed using the ImageJ software by an investigator blinded to experimental group assignments. Chronic brain atrophy was measured on NeuN-stained sections by subtracting the none-lesioned volume (NeuN-positive) of the ipsilateral cortex and striatum from that of the contralateral hemisphere in six brain slices (bregma 1.10 mm to -1.34 mm). The number of mature oligodendrocytes (APC⁺ cells), M1 microglia/macrophages (CD16/32⁺/Iba1⁺ cells), or M2 microglia/macrophages (CD206⁺/Iba1⁺ cells) was counted from 1–2 microscopic fields randomly selected from the peri-infarct area (within 300 μ m to the infarct). The width of the corpus callosum (CC) was measured on MBP-stained brain sections (bregma 0.5 mm) as previously described [29]. The width of the MBP-immunopositive CC area was measured every 160 μ m from the midline. MBP and SMI-32 fluorescence intensity were measured as described previously [26]. Briefly, 2 microscopic fields from the peri-infarct cortex and striatum, and 1 microscopic field from the peri-infarct CC were randomly selected from each brain and acquired using the same imaging settings. Images were then binarized and segmented under a consistent threshold (50%). The total black pixels in each image were then quantified.

Examination of recently proliferated cells

Recently proliferated cells were labeled with the S-phase marker 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, Missouri, USA) [18]. Briefly, BrdU was *i.p.* injected twice a day at a dose of 50 mg/kg body weight at 3–6 days after MCAO. At 28 days after MCAO, mice were sacrificed and coronal brain sections were prepared as described above. Sections were pretreated with 2N HCl for 1 h at 37°C followed by 0.1 M boric acid (pH 8.5) for 10 min at room temperature. Sections were then blocked with M.O.M. kit (Vector, Burlingame, CA, USA) for 1 h, and incubated with purified mouse anti-BrdU antibody (1:1000; BD Biosciences) for 1 h at room temperature and then overnight at 4°C. After a serial of washing, sections were incubated with the 488-AffiniPure donkey anti-mouse IgG (1:1000; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Fluorescence images were captured as described above. BrdU immunopositive cells were counted using ImageJ and expressed as the number of cells in the designated fields divided by the area (mm²). Oligodendrogenesis was evaluated on BrdU/APC double-stained sections. At least 4 microscopic fields were randomly sampled in each section.

Statistical analysis

All data are presented as mean $\pm$ SEM. The statistical differences among means of multiple groups were assessed by one- or two-way ANOVA followed by the Bonferroni *post hoc* test. The Pearson product linear regression analysis was used to correlate the multiple histological parameters and sensorimotor behaviors. A p value of less than 0.05 was deemed statistically significant.

Results

Delayed treatment of DHA and FO after ischemic stroke improves long-term histological and functional outcomes

The capability of improving long-term neurofunctional outcomes after stroke is imperative for a potential therapy to be translated from bench to bedside [30–33]. Our recent study revealed beneficial effects of post-stroke DHA injections combined with FO dietary supplementation against stroke-induced cognitive decline [25]. In the present study, we investigated whether the delayed DHA and FO treatments ameliorate long-term sensorimotor deficits after ischemic stroke (Fig. 1a). The cylinder test, which assesses spontaneous forelimb use of rodents, is sensitive in identifying stroke-induced sensorimotor asymmetry and suitable for evaluation of mild and chronic deficits [34]. MCAO induced prolonged asymmetric use of forelimbs in the cylinder test for up to 28 days in all groups of mice, for which an approximately 44% of spontaneous recovery was observed in vehicle-treated mice between day 3 and day 28 (Fig. 1b). Post-stroke DHA injections significantly reduced the asymmetric rate during the testing period (Fig. 1b; $p = 0.001$ vs. vehicle by two-way ANOVA). Notably, combined DHA and FO treatment demonstrated an even more potent beneficial effect than DHA injections alone (Fig. 1b; $p = 0.01$ DHA+FO vs. DHA by two-way ANOVA), which became prominent at day 9–28 after MCAO. In the rotarod test, MCAO caused motor deficits in vehicle-treated mice, which was prominent at 3 days after MCAO and largely recovered by 10 days after MCAO (Fig. 1c). Both DHA and DHA+FO treatments effectively reduced motor deficits at 3–10 days after MCAO (Fig. 1c; $p = 0.001$ DHA+FO or DHA vs. vehicle by two-way ANOVA). Compared to DHA injections alone, combined DHA+FO treatment showed significantly better efficacy (Fig. 1c; $p = 0.05$ DHA+FO vs. DHA by two-way ANOVA), perhaps as a result of the dramatically improved performance on day 10. The latency to fall of the mice receiving DHA+FO treatment gradually increased at 3–10 days after MCAO, reflecting motor learning during repeated tests.

We further examined whether the delayed and repeated n-3 PUFA treatments after stroke could reduce brain tissue loss in the gray and white matter. At 28 days after MCAO, brain atrophy determined on NeuN-stained coronal sections was significantly reduced in mice receiving combined DHA and FO treatment, compared to vehicle-treated mice, whereas DHA treatment alone had no effects (Fig. 1c,d). The number of surviving neurons in the peri-infarct cortical regions was increased after both DHA and DHA+FO treatments compared to control stroke mice, where combined DHA and FO treatment conferred significantly greater effect than DHA injections alone (Fig. 2e). In addition to the protection against injury in the gray matter, delayed n-3 PUFA treatment after stroke partially preserved

the integrity of white matter. The corpus callosum (CC), a structure rich in myelinated axons and vulnerable to ischemic injury [35, 36], displayed pathological changes in the gross morphology after MCAO, which is illustrated by the significantly reduced CC width (Fig. 2f). Importantly, post-stroke DHA treatment alone largely maintained the CC width at 28 days after MCAO; the combined treatment with FO showed no further effect (Fig. 2f–h).

Post-stroke DHA and FO treatments ameliorate white matter injury

We next investigated the microstructural changes of white matter components in the peri-infarct CC, cortex and striatum at 28 days after MCAO using histological indicators (Fig. 2a). Specifically, we evaluated the expression of MBP, a marker for myelin, together with immunohistochemistry using the SMI-32 antibody, which recognizes the nonphosphorylated epitope of neurofilament H, a marker of demyelination [37]. In sham-operated mice, MBP was abundantly expressed in the CC, cortex and striatum, whereas SMI-32 immunofluorescence was readily detectable in the cortex, but barely visible in the CC or striatum (Fig. 2b). The immunofluorescence of MBP and SMI-32 in the non-injured contralateral hemisphere showed similar patterns to that of the sham-operated non-ischemic mice, and n-3 PUFA treatments did not cause detectable alterations in the signal expression of MBP or SMI-32 (Supplementary Fig. 1). In the post-ischemic ipsilateral hemispheres, MCAO impaired the myelin sheath, which was visualized by a reduction of MBP and a concomitant increase of SMI-32 in all three regions examined in vehicle-treated mice (Fig. 2b). These pathological changes were quantified by measuring the ratio of SMI-32/MBP fluorescence intensity (Fig. 2c–e), an indicator of white matter injury and demyelination [14, 35]. Post-stroke DHA injections offered partial but significant protection against MCAO-induced myelin pathology in the CC, cortex and striatum (Fig. 2b–e). However, combined DHA and FO treatment almost abolished MCAO-induced elevations of SMI-32/MBP ratio, indicating a marked protective effect against white matter injury (Fig. 2b–e).

Combined DHA and FO treatment enhances post-stroke oligodendrogenesis

The improved white matter integrity observed at 28 days after MCAO in mice receiving n-3 PUFA treatments might result from reduction of white matter injury, and/or enhancement of white matter repair. After focal cerebral ischemia, regeneration of myelinating oligodendrocytes is crucial for remyelination, white matter restoration, and neurological recovery [2, 36]. To determine whether n-3 PUFA-associated protection of white matter involved an effect on oligodendrogenesis, we performed double-label immunostaining of BrdU and APC (marker for mature oligodendrocytes [38]) at 28 days after MCAO and quantified the numbers of APC⁺ and APC⁺/BrdU⁺ cells, respectively, in the peri-infarct CC, cortex, and striatum (Fig. 3a–c). In the non-injured contralateral hemisphere, oligodendrogenesis was barely detected at 28 days after MCAO (Supplementary Fig. 2). In the ipsilateral hemisphere, total oligodendrocytes (APC⁺ cells) were significantly increased in the peri-infarct cortex and striatum of mice receiving combined DHA and FO treatment, whereas DHA injections alone did not result in significant increases (Fig. 3e,f). In addition, DHA and FO combined treatment markedly enhanced post-MCAO oligodendrogenesis in the peri-infarct cortex and striatum, as evidenced by the increased number of mature oligodendrocytes that were expressing BrdU⁺/APC⁺ signals (Fig. 3h,i). Interestingly, although combined DHA and FO treatment did not enhance oligodendrogenesis significantly

in the CC (Fig. 3g), the total number of oligodendrocytes in the CC was augmented (Fig. 3d; DHA+FO 792.04 ± 42.38 cells/mm² vs. vehicle 617.28 ± 26.41 cells/mm², $p = 0.01$), suggesting that the increase of cell numbers in this region might result from the increased cell survival of oligodendrocytes rather than elevated oligodendrogenesis. These results suggest that DHA injections alone preserved the myelin sheaths against ischemic injury, while combined DHA and FO treatment also promoted oligodendrogenesis.

White matter integrity is linked to long-term sensorimotor recovery after stroke

The observed improvement of white matter integrity in n-3 PUFA-treated mice likely contributes to post-stroke sensorimotor recovery. We performed Pearson product linear regression analysis to assess the correlation between the white matter histological parameters and neurofunctional performance. The asymmetric rate in the cylinder test showed a significant and strong positive correlation with SMI-32/MBP ratio (Fig. 4a–c; CC $r = 0.738$, $p < 0.001$; cortex $r = 0.799$, $p < 0.001$; striatum $r = 0.748$, $p < 0.001$), suggesting that the preservation of white matter integrity by n-3 PUFA treatments may contribute to the improved sensorimotor recovery. We further examined whether post-stroke oligodendrogenesis was linked to the neurofunctional improvement, and the results revealed interesting topographical differences. In the CC, the total number of oligodendrocytes showed a moderate but statistically significant negative correlation with asymmetric rate in the cylinder test (Fig. 4d; $r = -0.481$, $p = 0.032$), whereas oligodendrogenesis was not associated with the sensorimotor deficits (Fig. 4g; $r = 0.124$, $p = 0.614$). In the cortex, the number of new born oligodendrocytes (Fig. 4h; $r = -0.706$, $p = 0.001$), but not the total oligodendrocytes (Fig. 4e; $r = -0.340$, $p = 0.132$), showed a strong negative correlation with the asymmetry rate. In the striatum, both total oligodendrocytes and newborn oligodendrocytes were moderately, but significantly linked to the levels of sensorimotor deficits (Fig. 4f,i; $r = -0.589$, $p = 0.008$ and $r = -0.575$, $p = 0.006$, respectively). In summary, these data indicate that the preservation of white matter integrity might causatively contribute to the improved sensorimotor recovery after stroke. However, the precise mechanisms, *e.g.* the relative contribution from the stimulated oligodendrogenesis *versus* oligodendrocyte protection, might differ in different brain regions.

Delayed DHA and FO treatments after stroke regulate microglia/macrophage polarization

To date, the effect of post-stroke n-3 PUFA treatment on microglial activation remains poorly understood. Ischemic stroke induces the polarization of microglia/macrophages, which may exert phenotypic-dependent impacts on tissue injury and repair [12, 39, 40]. After MCAO, an anti-inflammatory M2 phenotype is initially activated, which is transient and gradually overwhelmed by a persistent pro-inflammatory M1 phenotype [41]. We determined whether DHA injections or combined DHA and FO treatment influences microglial phenotypes by examining the expression of CD16/32 and CD206, markers for M1 and M2 microglia/macrophages, respectively [12]. Immunostaining was done at 14 days after MCAO, a stage when M1 microglia/macrophages peak and M2 microglia/macrophages subside [41]. In sham-operated mice, microglia displayed non-activated, ramified morphology in the CC, cortex and striatum, with low expression of Iba1 and extremely low to undetectable levels of CD16/32 or CD206 (Supplementary Fig. 3). In vehicle-treated mice at 14 days after MCAO (Fig. 5a), CD16/32 was abundantly expressed in Iba1⁺ microglia/

macrophages in the peri-infarct CC, cortex and striatum, consistent with previous reports [41]. DHA injections significantly reduced the numbers of CD16/32⁺/Iba1⁺ cells, in all three regions examined; combined DHA and FO treatment had no further effects (Fig. 5b–d). In contrast, CD206 expression was barely detectable in the CC, cortex and striatum of vehicle-treated mice 14 days after MCAO (Fig. 6a). While DHA injections alone caused a moderate increase of CD206⁺/Iba1⁺ cell numbers in the cortex and striatum, combined DHA and FO treatment robustly increased M2 microglia/macrophages in the CC, cortex, and striatum (Fig. 6b–d).

The phenotypic switch from M1 to M2 microglia elicited by n-3 PUFA treatments may contribute to the improved white matter integrity and sensorimotor recovery after stroke. In the CC, cortex, and striatum, the number of M1 microglia/macrophages at 14 days positively correlated with SMI-32/MBP ratio at 28 days after MCAO (Fig. 5e–g), whereas M2 microglia/macrophages negatively correlated with white matter injury (Fig. 6e–g). Interestingly, the number of M2 microglia/macrophages also showed significantly negative correlation with the asymmetric rate in the cylinder test (Fig. 6h–j); this correlation was absent for M1 microglia/macrophages (Fig. 5h–j). In summary, these data suggest that delayed and repeated treatment of DHA and FO after stroke was capable of modulating microglial polarization toward an anti-inflammatory M2 phenotype. Although both affected white matter integrity, M1 and M2 microglia/macrophages might play different roles in dictating the long-term sensorimotor functions. In particular, the M2 microglia/macrophages may be important for post-stroke neurological recovery.

Discussion

The present study is the first to investigate the therapeutic efficacy of delayed post-stroke n-3 PUFA treatment against long-term white matter injury and sensorimotor deficits. Our results demonstrated that significant and prolonged improvement of white matter integrity was achieved by combined post-stroke DHA and FO treatment, which led to improved sensorimotor recovery. Furthermore, the sustained white matter protection afforded by n-3 PUFAs is likely attributable to not only oligodendrocyte protection and enhanced oligodendrogenesis, but also a beneficial modulation of post-ischemia microglial responses.

White matter is vulnerable to ischemic/reperfusion insult. Therefore, the damage or insufficient repair of white matter contributes to the development of long-term neurological deficits [42]. On the one hand, ischemia/reperfusion induces cell death of the myelin-producing oligodendrocytes and, consequently, white matter demyelination [7]. On the other hand, the continuous presence of oligodendrocyte progenitor cells (OPCs) in the brain provides an opportunity for oligodendrocyte regeneration and white matter repair [2, 43, 44]. Within one week after ischemic injury, endogenous OPCs actively proliferate in the peri-infarct areas [45, 46], suggesting that white matter integrity might be partially reestablished by oligodendrogenesis and replenishment of the lost oligodendrocytes. In the present study, post-stroke DHA injections alone or in combination with FO dietary supplements led to better preserved white matter integrity, where in several parameters, combined DHA and FO treatment demonstrated more robust protection than DHA treatment alone. The beneficial effects by n-3 PUFAs were achieved possibly through the direct preservation of

oligodendrocytes or myelin sheaths, the enhancement of oligodendrogenesis and white matter repair, or both. DHA exerts potent protection against AMPA-induced cell death in cultured oligodendrocytes [26]. In addition, long-term elevation of brain n-3 PUFA levels by dietary supplementation or transgenic expression of a n-3 PUFA-generating enzyme potentiates post-ischemia oligodendrogenesis in rodent stroke models [18, 47]. An interesting finding of the present study is that n-3 PUFAs showed topographically different effects on oligodendrocytes in post-stroke brain. Specifically, in the cortex and striatum, DHA and FO combined treatment nearly doubled the number of new born oligodendrocytes compared to vehicle treatment, which likely accounted for the increased total oligodendrocytes (Fig. 3). In contrast, oligodendrogenesis in the CC was not altered following n-3 PUFA treatment despite the increased total number of oligodendrocytes (Fig. 3). These results suggested that in the CC, protection against oligodendrocyte cell death might be a major mechanism underlying the improved white matter integrity. Future studies are warranted to further investigate the contribution of various white matter injury and repair mechanisms to this observed topographical difference.

Another interesting finding is that different correlation patterns between oligodendrocytes and post-stroke sensorimotor recovery were observed in different brain regions (Fig. 4). In the CC, the total surviving oligodendrocytes but not the levels of oligodendrogenesis displayed a significant but moderate correlation with sensorimotor performance. In the striatum, both oligodendrogenesis and total oligodendrocytes moderately correlated with sensorimotor performance. In the cortex where the majority of corticospinal tract fibers originate, only the newly generated oligodendrocytes were strongly linked to the sensorimotor recovery. This difference might result from the anatomical and cellular composition of the CC, cortex and striatum, as well as indicates their relative contributions to sensorimotor functions. The observation that a significant amount of BrdU⁺ cells expressed the mature oligodendrocyte marker APC was to our surprise, as the failure of the newly generated OPCs to fully differentiate into mature, myelinating oligodendrocytes was thought to be a major obstacle that limits remyelination of the post-ischemic white matter [45, 48, 49]. Future functional assessment on the white matter, *e.g.* action potential transmission in the corpus callosum [26, 35], are needed to explore whether these regenerated mature oligodendrocytes would indeed lead to improved myelination of axons and nerve fiber conduction.

Inflammatory responses triggered by microglia/macrophages play an important role in the pathogenesis of stroke [50–52]. Ischemia/reperfusion rapidly activates microglia, which can exert different impacts on the injury and repair of gray and white matter, depending on their phenotypic polarizing state [12]. Under various pathological conditions such as ischemic stroke, traumatic brain injury or multiple sclerosis, M1 microglia/macrophages are generally considered to exacerbate oligodendrocyte cell death and destroy myelin through excessive proinflammatory responses [14, 27], whereas M2 microglia/macrophages facilitate remyelination and tissue repair [16, 36, 53]. A previous study using microglial cultures has shown that DHA and EPA both promote the M2 polarization of microglia by down-regulating M1 signature genes (*e.g.* TNF- α , IL-1 α , CCL5) and up-regulating M2 signature genes (*e.g.* CD206, TGF- β) [27]. DHA and EPA also inhibit the production and release of proinflammatory mediators, such as TNF- α and NO, from activated microglia [27].

Moreover, DHA and EPA enhance microglial phagocytosis [27], which would facilitate post-injury clearance of tissue debris. These effects of n-3 PUFAs on cultured microglia may, at least in part, explain the beneficial effects of n-3 PUFAs on microglial responses and white matter protection observed in the present *in vivo* study. After ischemia/reperfusion, the initial M2 microglial polarization is gradually overwhelmed by the destructive M1 polarization, which may lead to progressive tissue injury [41]. In mice receiving post-stroke n-3 PUFA treatments, we observed enhanced polarization of microglia/macrophages towards M2 at 14 days after MCAO. How exactly n-3 PUFAs modulate microglial responses remains largely unknown, although recent studies suggest a role of DHA-containing lipid bodies and their functional interplay with mitochondria [54, 55]. We have previously found that n-3 PUFAs enhance Akt signaling after hypoxia/ischemic brain injury [56]. While Akt is known to exert anti-inflammatory effects on microglia and macrophages [14], it remains to be determined whether n-3 PUFAs induce M2 polarization after stroke. Interestingly, although both M1 and M2 microglia strongly correlated with white matter integrity (Fig. 5e–g, Fig. 6e–g), only M2 microglia significantly correlated with post-stroke sensorimotor recovery (Fig. 5h–j, Fig. 6h–j). A similar correlation between M2 microglia and post-stroke functional recovery was previously noted in aged mice after distal MCAO [53]. In contrast, the M1 microglia correlated poorly with post-stroke sensorimotor deficits (Fig. 5). These results strongly implicate the importance of M2 microglia/macrophages in long-term tissue remodeling, thus promoting the microglia M2 polarization may be a rational therapeutic strategy after brain injury. A potential limitation of the present study is the use of immunohistochemical markers for the identification of M1 and M2 microglia. Since many cells may co-express both M1 and M2 markers, there could be some discrepancies between immunohistochemical results and the actual functional states of cells [57]. Nevertheless, the present study provides an initial screening of n-3 PUFA actions on microglia in relation to long-term stroke outcomes. Future studies on the functional evaluation of microglia phenotypes are strongly warranted.

To date, treatment to acute ischemic stroke remains largely limited to recombinant tissue-type plasminogen activator (tPA)-mediated endovascular thrombolysis. It is imperative for stroke research to develop therapies that have a wide treatment time window and ultimately lead to long-term neurological improvement [58–60]. While the protective effects of n-3 PUFAs against ischemic brain injury have been well established in the literature [18, 61], the majority of these previous studies were based on the preventative beneficial effect of n-3 PUFAs when delivered long before stroke onset. In studies that administered n-3 PUFAs within 1 h after post-ischemia reperfusion, neuroprotective effects were observed for up to 3 weeks after stroke [21, 23, 62]. In the current study, we extended the first injection of DHA to 2 h after reperfusion and further elevated brain n-3 PUFA levels in long term by FO dietary supplementation at 5 days after MCAO. Consistent with our recent finding that delayed DHA and FO treatments after MCAO promote cognitive recovery [25], the present study demonstrates long-term beneficial effect of n-3 PUFAs on white matter integrity and microglial responses. While DHA injections alone elicited some improvement in post-stroke histological and functional outcomes, such as improved sensorimotor recovery (Fig. 1), reduced neuronal death (Fig. 1), lessened white matter injury (Fig. 2), and promotion of microglia M2 polarization (Fig. 6), combination with FO dietary supplement demonstrated

greater beneficial effects on these parameters. This additive protection might be attributed to two potential mechanisms. Firstly, chronic supplementation of FO could have increased brain n-3 PUFA contents more persistently, compared to DHA injections alone. Secondly, other non-DHA components in the FO, *i.e.* EPA, might play a major role. EPA normally exists in extremely low levels in the brain but can be dramatically elevated after long-term FO supplement [18]. To facilitate the translation of n-3 PUFA treatment for clinical use, future studies should take into consideration the confounding effects from aging and stroke comorbidities [63–65], and also test the therapeutic efficacy in models involving tPA thrombolysis [66–69].

In summary, our study demonstrates that delayed administration of n-3 PUFAs as late as 2 h after ischemia/reperfusion promotes white matter restoration. Combining DHA injections with FO dietary supplementation significantly elevates post-ischemia oligodendrogenesis and modulates microglial responses toward the beneficial M2 phenotype, both of which correlate with improved long-term sensorimotor functions. Delayed DHA injections combined with FO dietary supplementation thus may be a promising therapy to achieve white matter protection in stroke patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Delayed treatment of DHA and FO after ischemic stroke elicits long-term improvement in sensorimotor function and gray and white matter integrity

Mice were subjected to 1 h of MCAO followed by DHA and FO treatments as described in *Methods*. **a** Illustration of the experimental timelines. Mice were pre-trained for the behavioral tests for 3 days before MCAO or sham operation. Two hours after MCAO, mice received DHA injections that lasted for 14 days. FO supplements were administrated 5–28 days after MCAO. Qd: once a day. Bid: twice a day. Sensorimotor functions were evaluated up to 28 days after MCAO by the cylinder and rotarod tests. Mice were sacrificed 14 or 28 days after MCAO for histological examinations. **b** The cylinder test was performed up to 28 days after MCAO and the asymmetric rate of the forelimb use was shown. **c** The rotarod test was performed up to 10 days after MCAO. *p 0.05, **p 0.01, ***p 0.001 by two-way ANOVA. n=6–9 mice per group. **d** Representative images of coronal brain sections showing

NeuN immunosignal (*red*) at 28 days after MCAO. *Dashed lines* illustrate the chronic brain infarct (NeuN-negative area). *Scale bar*: 1 mm. **e** The volume of tissue atrophy in the ipsilateral hemisphere. n=7 mice per group. *p 0.05, **p 0.01, ***p 0.001 by one-way ANOVA. **f** Representative images showing the morphology of the corpus callosum (CC) by MBP immunofluorescence at 28 days after MCAO. *Red boxes* indicate areas that were enlarged in the high-power images (the 2nd row). *Dashed lines* show the boundary of the CC. *Scale bar*: 1mm. **g** The width of the CC in the ipsilateral hemisphere measured every 160 μ m from the midline. *Shaded area* shows the levels that were illustrated in the bar graph in **h**. **h** Summarized data of CC width at 800 μ m and 960 μ m from the midline. n=6–9 mice per group. *p 0.05, **p 0.01 by one-way ANOVA.

Fig. 2. Post-stroke DHA and FO treatments mitigate white matter injury

a A representative image of MBP immunofluorescence in a coronal brain section at the level of bregma 0.5 mm at 28 days after MCAO. *Boxes* show the peri-infarct areas of corpus callosum (CC), cortex (CTX) and striatum (STR) where images from (**b**) were taken. *Scale bar*: 1 mm. **b** Representative images of MBP (*green*) and SMI-32 (*red*) immunofluorescence in the peri-infarct corpus callosum, cortex and striatum of the ipsilateral hemisphere, or the corresponding areas in sham-operated mice at 28 days after MCAO. *Scale bar*: 50 μ m. **c-e**. Quantification of the ratio of SMI-32 to MBP fluorescence intensity in the corpus callosum (**c**), cortex (**d**) and striatum (**e**) of the ipsilateral hemisphere. $n=6-9$ mice per group. ** p 0.01, *** p 0.001 by one-way ANOVA.

Fig. 3. Combined DHA and FO treatment after stroke enhances oligodendrogenesis

a A representative image of MAP2 immunofluorescence (green) in a coronal brain section at 28 days after MCAO. Boxes illustrate the peri-infarct areas from the corpus callosum (CC), cortex (CTX) and striatum (STR) where images in **c** were taken. Scale bar: 1 mm. **b** A representative image showing a newly generated and matured oligodendrocyte identified by double-label immunostaining of BrdU (green) and the mature oligodendrocyte marker APC (red). **c** Representative images showing double-label immunostaining of BrdU (green) and APC (red) in the peri-infarct corpus callosum, cortex and striatum at 28 days after MCAO. Boxes illustrate areas that were enlarged in the 4th column. Arrow: BrdU⁺/APC⁺ cell (yellow). Arrowhead: BrdU⁺ newly generated cell that is negative for APC signal (green).

Scale bar: 50 μm . **d–f** Quantification of total mature oligodendrocytes in the corpus callosum (**d**), cortex (**e**) and striatum (**f**). **g–i** Quantification of newly generated mature oligodendrocytes in the corpus callosum (**g**), cortex (**h**) and striatum (**i**). n=6–9 mice per group. *p 0.05, **p 0.01 by one-way ANOVA.

Fig. 4. White matter integrity correlates with sensorimotor recovery after ischemic stroke

Pearson product linear regression analysis was performed to correlate post-stroke white matter histological parameters with the mice's performance in the cylinder test. **a–c** Correlation of SMI-32/MBP ratio in the corpus callosum (**a**), cortex (**b**) and striatum (**c**) at 28 days after MCAO with the asymmetric rate of forelimb use in the cylinder test at 11–23 days after MCAO. **d–i** Correlation of total APC⁺ cell numbers (**d–f**) or BrdU⁺/APC⁺ cell numbers (**g–i**) in the corpus callosum, cortex and striatum at 28 days after MCAO with the asymmetric rate of forelimb use in the cylinder test at 11–23 days after MCAO. $n=6-7$ mice per group.

Fig. 5. Delayed DHA and FO treatments after stroke reduce microglia/macrophage M1 polarization

a Representative images showing double-label immunostaining of Iba1 (green) and CD16/32 (red) in the peri-infarct corpus callosum, cortex and striatum at 14 days after MCAO. Boxes indicate the regions that were enlarged in the 4th column. Arrow: Iba1⁺/CD16/32⁺ cell (yellow). Scale bar: 50 μ m. **b–d** Quantification CD16/32⁺/Iba1⁺ cells in the corpus callosum (**b**), cortex (**c**) and striatum (**d**). n=3–5 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle by one-way ANOVA. **e–g** Pearson correlation between CD16/32⁺/Iba1⁺ cell numbers at 14 days after MCAO and SMI-32/MBP ratios at 28 days after MCAO in the corpus callosum (**e**), cortex (**f**) and striatum (**g**). **h–j** Pearson correlation between CD16/32⁺/Iba1⁺ cell numbers in the corpus callosum (**h**), cortex (**i**) and striatum (**j**) at 14 days after MCAO and the asymmetric rate of forelimb use in the cylinder test at 11–23 days after MCAO. n=3–5 mice per group.

Fig. 6. Delayed DHA and FO treatments after stroke promote microglia/macrophages M2 polarization

a Representative images showing double-label immunostaining of Iba1 (green) and CD206 (red) in the peri-infarct corpus callosum, cortex and striatum at 14 days after MCAO. Boxes indicate the regions that were enlarged in the 4th column. Arrow: Iba1⁺/CD206⁺ cell (yellow). Scale bar: 50 μ m. **b–d** Quantification CD206⁺/Iba1⁺ cells in the corpus callosum (b), cortex (c) and striatum (d). n=3–5 mice per group. *p < 0.05, ***p < 0.001 by one-way ANOVA. **e–g** Pearson correlation between CD206⁺/Iba1⁺ cell numbers at 14 days after MCAO and SMI-32/MBP ratios at 28 days after MCAO in the corpus callosum (e), cortex (f) and striatum (g). **h–j** Pearson correlation between CD206⁺/Iba1⁺ cell numbers in the corpus callosum (h), cortex (i) and striatum (j) at 14 days after MCAO and the asymmetric rate of forelimb use in the cylinder test at 11–23 days after MCAO. n=3–5 mice per group.