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Maternal omega-3 fatty acid supplementation protects against lipopolysaccharide-induced white matter injury in the neonatal rat brain

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***Objectives*: Periventricular leukomalacia (PVL) is the predominant form of brain injury in premature infants, and no specific treatment currently exists for this condition. We have evaluated whether maternal omega-3 fatty acid (ω3 FA) treatment reduces endotoxin-induced PVL in the developing rat brain. *Methods*: Wistar rats with dated pregnancies were fed a standard diet or a diet enriched in ω3 FA (70% docosahexaenoic acid + 30% eicosapentaenoic acid mixture) during gestation. Intraperitoneal injection of lipopolysaccharide (LPS) was administered consecutively on the 18th and 19th embryonic days to establish the endotoxin-induced PVL rat model. The animals were divided into four groups: (i) control, (ii) PVL, (iii) PVL+low-dose ω3 FA and (iv) PVL+high-dose ω3 FA. At day P7, apoptosis and hypomyelination in periventricular white matter were evaluated by immunohistochemical assessments. *Results*: High-dose maternal ω3 FA treatment reduced brain weight loss. Maternal ω3 FA treatment given either in low or high doses greatly decreased caspase-3 immunoreactivity and increased myelin basic protein immunoreactivity, indicating a decrease in apoptosis and hypomyelination. *Conclusion*: Considering that no specific treatment is available for PVL, maternal ω3 FA supplementation may provide a nutritional strategy to limit periventricular white matter damage caused by infections during pregnancy.**

Keywords: apoptosis, DHA, EPA, newborn, periventricular leukomalacia

Introduction

Periventricular leukomalacia (PVL), involving focal white matter necrosis, diffuse gliosis and subsequent hypomyelination, is the predominant form of brain injury and the leading cause of chronic neurological morbidity in premature infants [1]. The most commonly postulated mechanisms underlying PVL pathophysiology are hypoxia–ischemia (H/I)-induced glutamate excitotoxicity, free-radical injury and inflammation [2,3].

Oligodendrocytes (OLs) are the principal cellular components in the white matter, and the developing OL (pre-OL) is a key cellular target that populates developing white matter during the period of greatest risk for PVL [4–6].

Current clinical practice for preventing PVL is as yet insufficient, and new approaches are needed to diminish PVL and the subsequent neurologic disability in premature infants. Because glutamate, free radicals, and proinflammatory cytokines play a role in the pathophysiology of PVL, prevention of free radicals and inflammatory mediators from killing pre-myelinating oligodendroglia is a potential method of PVL therapy [7].

It is well known that omega-3 fatty acids (ω3 FAs) are beneficial for brain function. Docosahexaenoic acid [22:6(n-3)] (DHA) and eicosapentaenoic acid [20:5(n-3)] (EPA) are the two major components of the ω3 FA family. The embryo cannot produce its own ω3 FAs and therefore is dependent on maternal supply [8]. Poor maternal DHA status increases the risk of inadequate DHA to support brain and retinal development, delaying or limiting neural and visual system development [9]. Depletion of dietary DHA is associated with adverse neurological outcomes in animals, suggesting that variations in maternal long-chain polyunsaturated fatty acid (PUFA) stores have the potential to affect fetal development [10]. Although the exact mechanisms of the neuroprotective action of DHA are not completely known, it has a role in (i) neurogenesis, neuronal differentiation, neurotransmission and synaptogenesis [11], (ii) inhibition of proinflammatory cytokine secretion [12,13], (iii) inhibition of apoptosis by suppression of excitotoxicity via blockage of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-kainate-type glutamate receptor [14] and activation of the ERK/MAPK pathway [15] and (iv) inhibition of free radical toxicity [16–18].

ω3 FAs accumulate rapidly in the central nervous system during the last intrauterine trimester, and preterm infants are born with limited ω3 FA stores [19,20]. Therefore, deficits of the ω3 FA stores experienced by preterm infants might aggravate the brain injury associated with PVL.

In the present study, we investigated whether (i) maternal DHA and EPA treatment during pregnancy has anti-apoptotic effects against endotoxin-mediated perinatal white matter injury (WMI) and (ii) DHA+EPA treatment decreases hypomyelination in endotoxin-mediated perinatal WMI.

Methods

Animals and drugs

This study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by

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the Animal Care and Use Committee of the Dokuz Eylul University, School of Medicine. Rats were housed in a temperature- and humidity-controlled animal facility with a 12-h dark:light cycle. Timed-pregnant Wistar rats were used, and the presence of sperm in the vaginal smear was considered as day 0 of pregnancy. All pregnant females had free access to food and water throughout the experiments. They were divided into four groups. Diet modifications were started from the second day of the pregnancy until birth, which occurs normally on the 21st day. Newborn rats were nursed by their mothers.

Standard rat chow (including 16% crude protein, 8.3% sugar, 9% crude cellulose, 3.5% crude fat), which does not contain ω3 polyunsaturated fatty acids, was purchased from TARIS (Agriculture Company, Izmir, Turkey). Its fatty acid composition is as follows: 45% saturated, 40% monounsaturated, 15% n-6 polyunsaturated fatty acids and 0% ω3 polyunsaturated fatty acids.

A standardized fish oil formulation (GNC, Los Angeles, CA, USA) was used. Each capsule (0.5 mL) contained 250 mg of DHA and 100 mg of EPA. In the high-dose ω3 FA treatment group, a constant volume of 0.4 cc per 100 g body weight was given daily by gavages. Under this condition, DHA and EPA concentrations were 1000 and 400 mg/kg, respectively. In the low-dose ω3 treatment group, a constant volume of 0.1 mL/100 g body weight was administered daily by gavages. The doses of DHA and EPA corresponded to 250 and 100 mg/kg in the low-dose group. ω3 FA doses were chosen according to the previous studies on rat models [21–25]. As olive oil contains antioxidants such as vitamin E and carotenoids, which potentially affect the results, we used equal amounts of olive oil for all groups.

Stock lipopolysaccharide (LPS) suspension was sonicated, aliquotted, and stored at −70°C until use. Before use, the LPS stock suspension was sonicated for 15 s and then diluted with free saline to appropriate concentrations. To obtain live offspring for examination of neonatal brain injury, LPS (from *Escherichia coli*, serotype 055:B5, Sigma Chemical Company, St. Louis, MO, USA) was consecutively administered on gestation days 18 and 19 at a dose of 500 μg/kg. This dose schedule was selected on the basis of the results from previous studies [26].

Twenty-eight rat pups from four different dams were divided into four groups: (i) control (group 1, $n = 7$), (ii) LPS-administered group (group 2, $n = 7$), (iii) high-dose ω3 FA-supplemented and LPS-administered group (group 3, $n = 7$) and (iv) low-dose ω3 FA-supplemented and LPS-administered group (group 4, $n = 7$).

Rat pups delivered spontaneously were reared with their dams until the time of sacrifice at P7 (day of birth is day 1). Day P7 in the study was chosen because this period coincides with the peak of the brain growth spurt, and the neurogenetic events occurring at this time are comparable with those occurring in the human fetus during the 32nd to 34th week of pregnancy [27].

Histomorphological and immunohistochemical assessment

On P7, rat pups were anesthetized with intraperitoneal thiopental (12 mg/animal); a butterfly needle (21 gauge) was inserted into the left ventricle and the right atrium was incised. Each animal was perfusion-fixed with 4% paraformaldehyde. After perfusion, the brains were removed and stored in the same fixative for at least 3 days. Brain tissues were processed by routine histological methods and embedded in paraffin blocks. All histomorphological analyses described below were performed by two investigators blinded to the rat's treatment. The brain sections were taken using a rotary microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany) with disposable metal microtome blades (Type N35, Feather Company, Osaka, Japan) to obtain serial sections in a coronal plane from the paraffin block of tissues. Serial coronal sections of 6–8 μm thickness were obtained. Three consecutive coronal sections through the periventricular area that corresponded approximately to plate nos. 13-15-17 were defined and taken in accordance with the atlas of Paxinos and Watson [28]. A physical dissector was used for the stereologic method [29]. The sections were collected on lysine-coated slides and baked at 60°C overnight. Tissue sections were deparaffinized and hydrated through xylene and graded alcohol series; endogenous peroxidase was inactivated in 3% hydrogen peroxide in methanol for 10 min at room temperature. Sections were washed with phosphate-buffered saline (PBS) followed by antigen retrieval with 10 mmol/L sodium citrate buffer (pH 6) in a microwave oven for 20 min. The Vectastain ABC Elite Kit P-6102 was used according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). After the sections were incubated with Vectastain-diluted normal blocking serum for 30 min at room temperature, excess serum from the sections was blotted. Then, the sections were incubated overnight at 4 C with the specific primary antibodies, anti-MBP monoclonal antibody 1:100 diluted in buffer anti-Myelin Basic Protein (anti-MBP mouse monoclonal antibody, SC-71546, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-caspase-3 antibody 1:5 diluted in buffer (rabbit polyclonal antibody, RB-1197-R7, Thermo Scientific, Fremont, CA, USA). On the following day, vectastain biotinylated immunoglobulin G (IgG) secondary antibody was applied for 30 min at room temperature. Sections were washed with PBS followed by incubation with Vectastain Elite ABC Reagent for 30 min. Than sections were washed again with PBS followed by chromogenic detection with stable diaminobenzidine substrate (Roche Diagnostics, Mannheim, Germany) for 5 min at room temperature and counterstained with Mayer hematoxylin, cleared and mounted. Negative control samples, in which an equal amount of IgG was substituted for the primary antibody, were included in each assay and were found to be uniformly negative.

Image analysis methods

All available sections (at least three per brain) were analyzed; only sections with obvious technical artifacts related to the staining procedure were excluded. After the staining process had been completed, sections were examined and the images were analyzed by using a computer-assisted image analyzer system consisting of a microscope (Olympus BX-51, Tokyo, Japan) equipped with a high-resolution video camera (Olympus DP-71, Tokyo, Japan). All sections were digitally photographed.

Semi-quantification of immunostaining data

A grade system [6] was used to score the quantity of anti-caspase-3 and anti-MBP-positive staining in the sections. The score was defined as follows: 0 = no immunoreactivity; 1 = very little positive staining was observed, and the staining was mild; 2 = positive staining was moderate and between grade 1 and grade 3; and 3 = strong positive staining was evenly distributed across the whole image. To maintain consistency of scoring, each section was graded by two persons blinded to the treatments, and the average was taken. Digital microscopic images were taken at the area where the positive cells were observed for each brain section. The average of the scores was used to represent the grade of anti-caspase-3 and anti-MBP immunostaining for each brain.

Statistical analysis

Values are presented as mean ± SE. As the data obtained from the study were inconsistent with normal distribution, we used the non-parametrical statistical methods. The Kruskal–Wallis test was used for comparing the four groups. Differences between the two groups were examined with the Mann–Whitney *U*-test, with findings of $p < 0.05$ considered significant.

Results

In the present study, maternal weight gain during pregnancy and brain/body weight ratios of the offspring at P7 were evaluated. There was no significant difference between the four groups in terms of maternal weight gain during pregnancy. A significant reduction of brain/body weight ratio occurred in the PVL group compared with the control group. Maternal ω3 FA treatment with high dose, but not low dose, was shown to reduce brain weight loss (Figure 1).

Detection of apoptotic cells was performed utilizing the anti-caspase-3 immunostaining. A grade system [6] was used to score the quantity of anti-caspase-3 staining. Compared with the control group, LPS injection significantly increased the caspase-3-positive staining indicating apoptosis (Figures 2 and 3).

Prenatal maternal ω3 FA treatment given either in low or high doses decreased caspase-3 immunoreactivity. But the caspase-3 score was significantly lower in the control group than the ω3 FA-treated PVL groups ($p < 0.05$ for low and high ω3 FA doses). No significant difference was observed between low- and high-dose ω3 FA-treated PVL groups in terms of caspase-3 immunoreactivity (Figures 2 and 3).

LPS-induced hypomyelination was demonstrated by decreased MBP immunoreactivity. MBP staining was clearly detectable in the P7 control rat brain, primarily in the periventricular white matter tract (Figure 4). The same grade system [6] was used to score the quantity of anti-MBP positive staining. Compared with the control group, LPS injection in the rat brain significantly reduced the MBP-positive staining (Figure 5).

Maternal prenatal ω3 FA treatment greatly prevented LPS-stimulated loss of MBP staining in both treatment doses. No significant difference was observed between low- and high-dose ω3 FA-treated PVL groups in terms of MBP-positive staining (Figure 5).

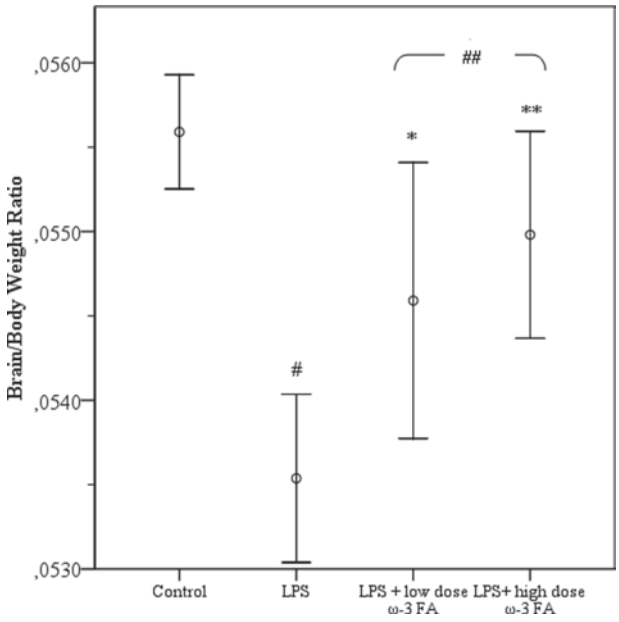

Figure 1. Comparative brain/body weight ratio of the groups. Data are expressed as mean ± SE, $n = 7$ per group, $^{\#}p = 0.009$ versus control; $^{*}p > 0.05$ versus lipopolysaccharide (LPS) group; $^{**}p = 0.017$ versus LPS group; $^{\#\#}p > 0.05$. ω3 FA, omega-3 fatty acid.

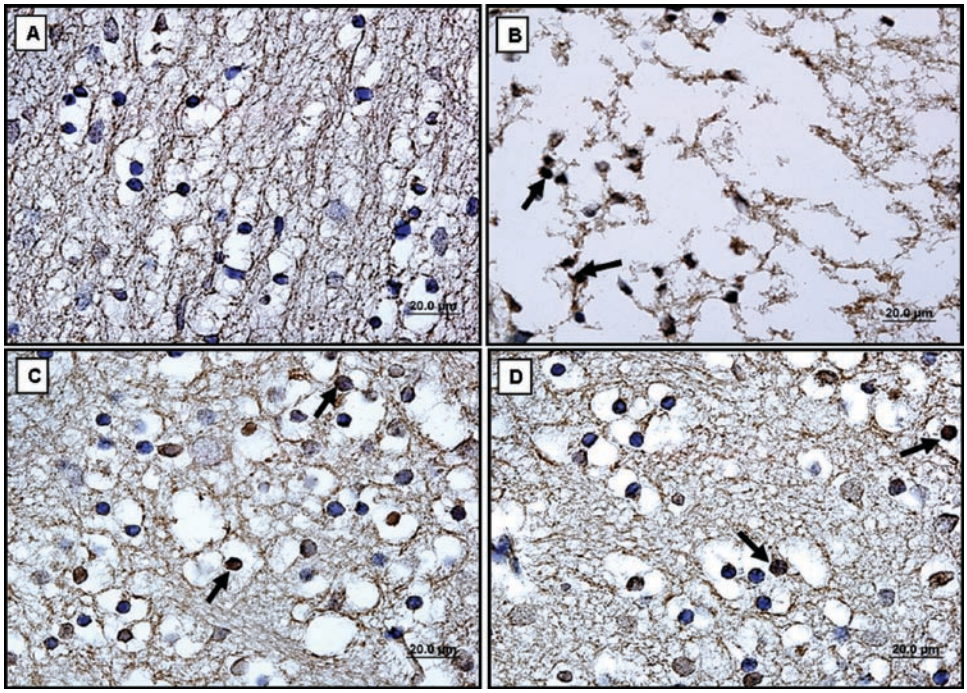

Figure 2. Representative light-microscopic images of anti-caspase-3 immunohistochemically labeled oligodendrocyte cells in the periventricular region in the control group (a), the group treated with lipopolysaccharide (LPS) (b), the group treated with low-dose omega-3 fatty acids (ω3 FAs) (c) and the group treated with high-dose ω3 FAs (d). Anti-caspase-3 immunopositive cells were significantly enhanced in the LPS group (b) whereas the groups treated with ω3 FAs showed a reduced number of anti-caspase-3 immunopositive cells at the periventricular region (c, d) when compared with the control group. Arrows (→) indicate anti-caspase-3 immunopositive cells.

Discussion

DHA is the most abundant essential ω3 FA present in the brain, and it is not only a structural component, but, more importantly, a modulator of important biological processes. Principal bases of our hypothesis were the instrumental role of DHA on fetal brain development and the poor DHA status of preterm infants because DHA is mostly transferred to the fetus by the placenta in the third trimester. Our results suggested the neuroprotective role of maternal DHA supplementation against LPS-induced periventricular WMI by demonstrating decreased caspase-3 activities and hypomyelination.

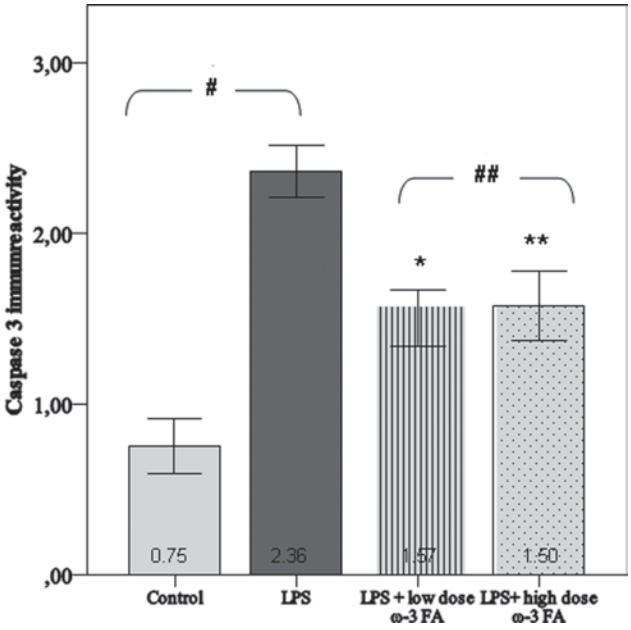

Figure 3. The effect of maternal omega-3 fatty acid (ω3 FA) treatment on lipopolysaccharide (LPS)-induced apoptosis in the periventricular region of the rat brain: caspase-3 immunoreactivity. Results are expressed as means ± SE of seven pups from four different dams. $^{\#}p = 0.002$, $^{*}p = 0.011$ versus LPS group; $^{**}p = 0.003$ versus LPS group; $^{\#\#}p > 0.05$.

Therapeutic uses of ω3 FAs in maintaining and developing brain health is under active investigation. Recently, maternal DHA-enriched diet during pregnancy was found to provide neuroprotection against H/I brain injury, by inhibiting oxidative stress and apoptotic neuronal death in a newborn rat model [16]. In addition, DHA treatment given either prior to or following the H/I insult in 7-day-old rat pups was found to improve functional outcomes in H/I perinatal brain injury [30,31].

In the present study, protection of MBP by maternal DHA treatment was demonstrated as indicated by morphology and the higher staining of MBP-positive cells in the newborn rat brain following maternal LPS injection at P7. Potential mechanisms whereby DHA might provide neuroprotection against pre-OL injury and subsequent myelin loss in LPS-induced periventricular WMI principally include inhibition of the apoptotic and proinflammatory signaling cascades stimulated by LPS administration. One of the well-understood mechanisms of DHA neuroprotection is its anti-inflammatory action. LPS used in this study activates its receptor, toll-like receptor (TLR) 4, then initiates the activation of downstream signaling cascades leading to the activation of nuclear factor κB (NF-κB) and MAPK and to the expression of proinflammatory cytokines [32]. Unsaturated fatty acids have been demonstrated to inhibit TLR4 [33], and action of all TLRs have been shown to be inhibited by DHA [34]. DHA may exert anti-inflammatory effects by altering the presentation of TLR4 on the microglia cell membrane, thereby modulating

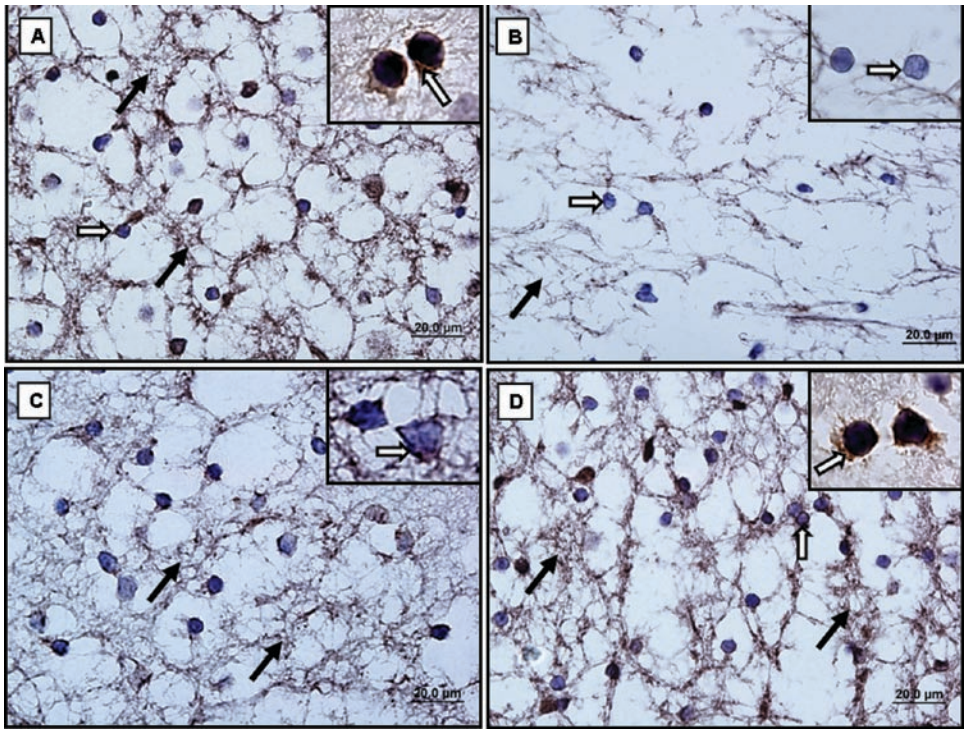

Figure 4. Representative light-microscopic images of MBP immunoreactivity with labeled oligodendrocyte cells and myelinated fibers in the periventricular region in the control group (a), the group treated with lipopolysaccharide (LPS) (b), the group treated with low-dose omega-3 fatty acids (ω3 FAs) (c) and the group treated with high-dose ω3 FAs (d). Labeled sections with MBP immunohistochemistry showed normal myelination and significantly immunopositive oligodendrocytes in the control group (a), whereas in the LPS group, reduced immunopositive oligodendrocytes were seen at the periventricular region. The groups treated with ω3 FAs showed significantly high anti-MBP immunopositive oligodendrocytes at the periventricular region (c, d) when compared with the LPS group (b). White arrows indicate anti-MBP immunopositive cells (a, c and d). In (b), there is no MBP immunreactivity. Black arrows indicate myelination in the periventricular region. The myelin fiber organization is disorganized in the LPS group.

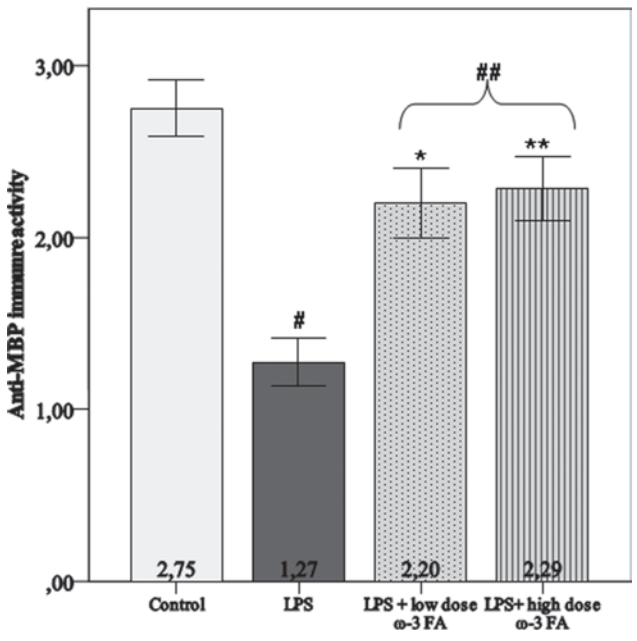

Figure 5. The effect of maternal omega-3 fatty acid (ω3 FA) treatment on lipopolysaccharide (LPS)-induced hypomyelination in the periventricular region of the rat brain: MBP immunoreactivity. Results are expressed as means ± SE of seven pups from four different dams. $^{\#}p = 0.003$ versus control, $^{*}p = 0.003$ versus LPS group; $^{**}p = 0.002$ versus LPS group; $^{\#\#}p > 0.05$.

the cyclooxygenase-2 signaling pathway and reducing pro-inflammatory cytokine production [35].

Apoptosis has been reported as a contributing mechanism for cell death in WMI. Furthermore, microglial activation and apoptosis of OL precursors occur in the gray matter and white matter 2–9 days after LPS administration [36]. Although we have not demonstrated the anti-apoptotic signaling mechanisms activated by DHA, our results showed that caspase-3-staining positive cells were decreased in the periventricular white matter at P7 by maternal DHA-enriched diet. Caspase activation plays a central role in apoptosis, and caspase-3 appears to be an especially important effector enzyme in neuronal cell apoptosis. In a previous study, it was suggested that DHA exclusively activated the ERK/MAPK pathway, then promoted the early expression of anti-apoptotic Bcl-2, decreased the pro-apoptotic Bax expression and reduced caspase-3 activation [15]. Another possible mechanism leading to decreased apoptosis by DHA treatment in our study is the inhibition of glutamate-induced excitotoxicity by DHA treatment. Previous studies suggested a role for glutamate receptors and glutamate transporters in PVL, and neuroprotective effects of treatments based on glutamate receptor blockage were demonstrated in rodent models of PVL [37–39]. There have been strong evidences that DHA has an inhibitory effect on AMPA-kainate-type glutamate receptors. It was observed that excitotoxicity triggered by an AMPA receptor agonist was markedly reduced in hippocampal slices treated with DHA, and the results indicated that the neuroprotective actions of DHA could result from the downregulation of AMPA receptors in the hippocampal membranes [14].

Although we did not evaluate the long-term functional effects of ω3 FA therapy, results from the previous studies indicate that ω3 FA supplementation during brain development improves functional outcomes [30,31]. It has been found that maternal ω3 PUFA supplementation significantly reduced brain damage and improved long-term neurological outcomes up to 5 weeks after neonatal H/I injury. The neuroprotective effects conferred by ω3 PUFA supplementation has been attributed to its anti-inflammatory action by inhibiting NF-κB activation and subsequently releasing inflammatory mediators [40].

The effects of EPA and DHA are not differentiated in most studies. Comparative studies have shown that DHA is more potent than EPA in terms of anti-inflammatory and antioxidant actions [41,42]. EPA has been found to be the precursor for resolvin E1, which is a lipid mediator that plays a major role in reducing (i) the response by regulating leukocyte extravasation through cell surface adhesion, (ii) leukocyte rolling and (iii) platelet aggregation [43]. EPA is detected as a trace component in the brain and has been recognized as one of the precursor fatty acids of DHA. It is not clear whether EPA has neuroprotective effects directly or by increasing the DHA content of the brain. Recently, it has been found that EPA protects against neurodegeneration by modulating synaptic plasticity and activating the PI3-kinase/Akt pathway, possibly by its own functional effects in neurons and glial cells and by its capacity to increase brain DHA [44]. The similarities between our data and the data from other studies using pure DHA suggest that the neuroprotective effects of ω3 FA combination on LPS-induced apoptosis in the present study may be attributable to the action of DHA in the central nervous system to a great extent. However, the possible role of EPA that was present in the fish oil formulation used here cannot be ruled out.

The main limitations of this preliminary study are that we did not explore molecular mechanisms and did not evaluate the long-term functional improvements. Additionally, measuring DHA and EPA levels in the brain tissue would be helpful to make a correlation between brain ω3 FA levels and apoptosis. Further detailed studies with different doses and different administration methods of ω3 FA therapy will be necessary in order to better understand the effects of ω3-enriched diet on PVL.

In summary, our results suggest that ω3 FA-enriched diet during pregnancy provides neuroprotection by inhibiting apoptotic neuronal death and hypomyelination in LPS-induced periventricular WMI. Because of the fact that no specific treatment currently exists for premature infants with PVL and the majority of infants with PVL can be identified only after the damage, nutritional supplementation of maternal diets with ω3 FAs is potentially important as a protective therapy.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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