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Docosahexaenoic acid reduces cerebral damage and ameliorates long-term cognitive impairments caused by neonatal hypoxia-ischemia in rats

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Abstract

As the interest in the neuroprotective possibilities of docosahexaenoic acid (DHA) for brain injury has grown in the recent years, we aimed to investigate the long-term effects of this fatty acid in an experimental model of perinatal hypoxia-ischemia in rats. To this end, motor activity, aspects of learning and memory function and anxiety, as well as corticofugal connections visualized by using tracer injections were evaluated at adulthood. We found that in the hours immediately following the insult, DHA maintained mitochondrial inner membrane integrity and transmembrane potential, as well as the integrity of synaptic processes. Seven days later, morphological damage at the level of the middle hippocampus was reduced, since neurons and myelin were preserved and the astroglial reactive response and microglial activation were seen to be diminished. At adulthood, the behavioral tests revealed that treated animals presented better long-term working memory and less anxiety than non-treated hypoxic-ischemic animals, while no difference was found in the spontaneous locomotor activity. Interestingly, hypoxic-ischemic injury caused alterations in the anterograde corticofugal neuronal connections which were not so evident in rats treated with DHA. Thus, our results indicate that DHA treatment can lead to long-lasting neuroprotective effects in this experimental model of neonatal hypoxia-ischemic brain injury, not only by mitigating axonal changes but also by enhancing cognitive performance at adulthood.

Keywords:

hypoxia-ischemia; neuroprotection; docosahexaenoic acid; neuronal connection; cognitive deficit; mitochondrial injury.

Introduction

With an incidence of 2–6/1000 term births [1], perinatal hypoxia-ischemia (HI) is a major cause of death and long-term disability worldwide, due to the frequent occurrence of irreversible brain damage and subsequent loss of neuronal function which lead to cognitive impairment and some partial motor dysfunction [2-5]. Here, we have investigated the neuroprotective effects of docosahexaenoic acid (DHA) in experimental HI encephalopathy. DHA is a long-chain omega-3 fatty acid, commonly found in fish such as salmon and tuna. It is an essential dietary fatty acid and its deficiency is associated with visual disorders and biochemical changes in the brain [6-10]. In fact, DHA is the major polyunsaturated fatty acid in the adult mammalian brain, where it constitutes more than 30% of the total phospholipid content of cellular membranes. Since DHA provides plasma membrane fluidity at synaptic regions, it is crucial for maintaining membrane integrity and, consequently, neuronal excitability and synaptic function [7, 11-13].

The newborn brain is highly at risk of oxidative imbalance, due to its high utilization of oxygen, its relatively poorly developed antioxidant defense, its higher concentrations of free iron and its high amount of easily oxidizable fatty acids [14-16]. When membrane lipoproteins and polyunsaturated fatty acids suffer attacks from free radicals, many oxygenated compounds are produced. Since antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged, by removing free radical intermediates and by inhibiting the oxidation of other molecules, exogenous DHA antioxidant therapy can in principle be a powerful tool in order to diminish cellular damage when perinatal hypoxia-ischemia occurs.

Therefore, the main goal of the present work was to analyze the long lasting neuroprotective effects of DHA administration by specifically evaluating cognitive impairments and the neuronal connections. As a key player in learning and memory adjustment, the hippocampus participates in integrating transferred outside information into the nerve center [17-19]. It is also responsible for spatial memory and plays important

roles in cognition and these functions of the hippocampus rely on its plasticity [20]. Similarly, the striatum and prefrontal cortex, in particular the medial prefrontal cortex, are heavily involved in the processing of working memory, [21]. Typically, cognitive impairments are associated with tissue loss in the hippocampus and cortex areas [22].

To this end, we morphologically assessed 14 day old (medium term) rat brains to evaluate damage to neurons, oligodendrocytes, astrocytes and microglia. Since we observed evidence of treatment effectiveness at this stage, we then analyzed the mitochondrial state, because mitochondria are known to be involved in the cascade triggered after hypoxia-ischemia, as well as the expression of synaptophysin and spinophilin, genes known to be implicated in synaptic transmission, immediately after (0 h), 3 h and 12 h after (short term) the HI event. Finally, we carried out behavioral studies and neuronal tracing experiments in adulthood to assess the long-term effects of DHA on cognitive function (long term).

Material and methods

Animals and HI procedures

Sprague-Dawley rats were used in all surgical and experimental procedures which were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the European Communities directive 2010/63/EU regulating animal research. All the protocols employed were previously approved by the Committee on the Ethics of Animal Experiments of the University of Basque Country (UPV/EHU) (Permit Number: CEEA/ 341-344/2014/ALVAREZDIAZ). Animals were maintained and experiments were conducted in accordance with ARRIVE (Animal Research: Reporting In Vivo Experiments). All efforts were made to minimize suffering (all surgery was performed under anesthesia) and to avoid unnecessary animal testing.

The hypoxic-ischemic brain injury was induced in perinatal rat pups on postnatal day 7 (P7) by the Rice-Vannucci method [23]. Under isoflurane anesthesia administered at 3% for induction and 1.5% for maintenance, the left common carotid artery was permanently ligated at two locations with 6-0 surgical silk and then cauterized to block blood flow through the ipsilateral carotid circulation. Following a 2-h recovery period, rats were exposed to humidified 8% oxygen in a nitrogen gas mixture with a flow of 5 l/min for 135 minutes, while being maintained at 36 °C. After hypoxic exposure, pups were returned to their biological

mothers until they were euthanized at the different times of study. If rats showed signs of increased or decreased respiratory rate, decrease of activity, loss of appetite, isolation from littermates, which may indicate pain or distress in the animal, they were euthanized by trained personnel of the animal unit of the University of Basque Country using sodium pentobarbital (in neonates) or carbon dioxide (after weaning); but no animals showed any signs of pain. After weaning, animals were housed in individual stalls, and maintained in a climate-controlled environment on a 12-hour light/dark cycle where they had free access to food and water.

Experimental groups

Pups were randomly assigned to three experimental groups: control rat pups had neither common carotid artery ligation nor a period of hypoxia (Control); the hypoxic-ischemic injured group (HI); and another group of HI rats who received a single dose of 1 mg/kg DHA injected intraperitoneally [24] 10 minutes before hypoxia (HI+DHA). DHA (Sigma-Aldrich Co. Ltd., Gillingham, UK) was dissolved in %25 human serum albumin (Sigma Chemical Co) diluted in normal saline. We decided to use this dose of DHA after histologically evaluating the effects of two concentrations (1 mg/kg vs. 3 mg/kg); no statistically significant differences in neuropathology were found between these doses (data not showed). A control+vehicle and HI+vehicle groups were established to test the response to the vehicle, but were not found to be different with respect to the control and HI groups, respectively.

Morphological evaluation

Tissue processing

Tissue for the three experimental groups was morphologically evaluated on postnatal day 14 (P14), seven days after the HI insult: Control ($n \geq 5$), HI ($n \geq 8$) and HI+DHA ($n \geq 8$). Animals were sacrificed with a sodium pentobarbital overdose and transcardially perfused with saline-heparin followed by cold 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate-buffered saline (PBS) (pH 7.3). Brains were removed and immersed in the same fixative at 4°C overnight. Some of the brains were then dehydrated with graded ethanol and xylene, embedded in paraffin wax and cut in 5 µm coronal sections using a microtome (1150 Autocut, Reichert-Jung). Sections were cut at the level of interaural distance 5.40 mm and bregma -3.60 mm level, according to the Paxinos and Watson atlas [25] and then stained with Nissl (cresyl violet).

Assessment of infarct area

To assess the severity of tissue injury, the extent of infarction in coronal sections stained with cresyl violet (Sigma-Aldrich Co.) and tolonium chloride (Sigma-Aldrich Co.) was first photographed (Carl Zeiss Stemi 2000-C steromicroscope). Then, measurements were performed by a researcher who was blind to the conditions of the treatment using the ImageJ software (public domain, National Institutes of Health). The area of infarction was defined as the loss of the normal cresyl violet staining pattern using the formula (C-I/C)*100, where C is the mean of the contralateral area and I is the mean value of the ipsilateral area for each brain sample.

Histopathological Measurements

The histopathology of the different groups was evaluated by analyzing the CA 1, CA 2-3 and dentate gyrus (DG) of the hippocampus and the parietal cortex (CTX), with an Olympus BX 50 light microscope (x400). Histopathological scoring of cresyl violet stained sections was done blindly as previously described [26]: injury in the parietal cortex was graded from 0 to 4, and in each region of the hippocampus, in the CA 1, CA 2-3 and DG areas, from 0 to 6 points, so that the maximum score for the hippocampus was 18. Total brain injury score could reach 28 points, the sum score for all four regions and macroscopic evaluations.

Immunohistochemical analyses

White matter injury

Myelination was evaluated by using the myelin binding protein (MBP) immunohistochemistry. 5 µm coronal sections were dewaxed, rehydrated, washed two times in PBS and treated for 15 minutes with H_2O_2 (1%) in PBS to inactivate endogenous peroxidases. After being rinsed thoroughly in PBS to completely eliminate H_2O_2, the sections were incubated for 10 minutes in a blocking solution (0.25% Triton X-100 in PBS) and washed twice in 0.5% BSA in PBS. Next, sections were incubated overnight at 4°C with monoclonal mouse primary antibody to MBP (1:100, Santa Cruz Biotechnology, CA, USA) diluted in 0.25% Triton X-100 and 0.5% BSA in PBS. After immunostaining, the slides were washed three times in PBS and incubated for 1 hour with peroxidase-labeled anti-mouse second antibody at a dilution of 1:100 (HRP anti-mouse, Santa Cruz Biotechnology, CA, USA). Finally, the sections were stained with

diaminobenzidine (DAB), counterstained with hematoxylin (Sigma-Aldrich), dehydrated in ethanol, cleared in xylene, and coverslipped with DPX mounting medium (Sigma-Aldrich). Immunolabeled sections of different areas of the brain were analyzed using an Olympus BX 50 light microscope.

Assessment of white matter injury was performed by measuring the density of MBP immunostaining, using Image J software as previously described [26-27]. Unaltered TIFF images were digitized, segmented (using a consistent arbitrary threshold of -50%), and binarized (black versus white). The total number of black pixels per hemisphere was counted, and average values were calculated per brain, and expressed as pixels per hemisphere. Hemisphere areas were also outlined and measured for each section that was analyzed by densitometry. At least three sections per brain were analyzed and only sections with technical artifacts related to the staining procedure were excluded. Densitometric values were expressed as ratios of ipsilateral-to- contralateral hemispheric measurements (I:C).

Astrogliosis and microglial activation

Reactive astrogliosis was assessed using glial fibrillary acidic protein (GFAP) immunochemistry and microglia were visualized using the calcium binding adaptor molecule 1 (IBA-1), which is a microglia/macrophage-specific calcium-binding protein. To this end, perfused brains were carefully removed and post-fixed overnight, equilibrated in 30% sucrose in 0.1 M phosphate-buffered saline and left at 4 °C. 60-µm coronal sections at interaural distance 5.40 mm and bregma -3.60 mm level were obtained using a vibratome (Leica 1325 Biosystems). After washing three times in PBS, a blocking solution (0.25% Triton X-100 in 0.5% BSA in PBS) was applied for 15 minutes. Sections were incubated overnight at 4°C with a polyclonal rabbit primary antibody to GFAP (1:1000, Dako, Denmark) or with a polyclonal goat primary antibody to IBA-1 (1:1000, Abcam, Cambridge, UK) both diluted in 0.25% Triton X-100 in 0.5% BSA in PBS. The slides were washed three times in PBS and incubated for 1 hour in an anti-goat or anti-rabbit secondary antibody conjugated with Alexa 488 (1:200; Invitrogen, The Netherlands). The IBA-1 slices were also counterstained with DAPI (1:1000; Invitrogen). Negative controls received the same treatment omitting the primary antibodies and showed no specific staining. Images from the CA 1 and dentate gyrus areas of the hippocampus were taken with an Olympus Fluoview FV500 Confocal Microscope.

Flow cytometry analysis

Tissue collection

For the assessment of mitochondrial state by flow cytometry, three experimental groups (Control, HI and HI+DHA; n≥5) and three different points of time after H/I insult (0 h, 3 h and 12 h) were analyzed. Animals were sacrificed with pentobarbital sodium overdose and perfused with Ringer lactate solution. Ipsilateral brain sections were isolated and cut with a blade in a lactate solution always kept on ice cold and finally disaggregated in collagenase (Invitrogen) solution (1,5 mg/ mL) in Hanks' Balanced Salt solution (HBSS; Sigma-Aldrich, St Louis, Mo, EEUU) at 37 °C for 20 min by means of a cell strainer. Cell suspension was washed with HBSS by centrifugation at 1600 g for 5 minutes, and after removing the supernatant, the pellet was suspended in 5 mL HBSS. Then, 600-1000 mL of cell suspensions ($1x10^6$cells/mL) were incubated with different fluorochromes and conjugates. Analyses were determined by an EPICS ELITE Flow Cytometry (Colter, Inc., Miami, FL, USA). To exclude debris and cellular aggregates, samples were gated based on light scattering properties, in side scattering (SSC), which correlates with cell complexity, and forward scattering (FSC), which correlates with cell size, and 10,000 events per sample within a gate (R1) were collected. Events within R1, which corresponded to individual cells, were plotted for their fluorescence. We first analyzed unstained samples from each animal, serving as a negative control to correct for autofluorescence. Summit v4.3 software was used for data analysis.

Mitochondrial inner membrane integrity

Mitochondrial inner membrane integrity was analyzed by using the Nonyl Acridine Orange (NAO, Invitrogen) fluorochrome, which binds to cardiolipin that is located in the mitochondrial internal membrane and is essential for protein functionality and ATP synthesis. Cell suspensions were incubated with 4 µl NAO (10^{-2} M) in PBS at 4 °C and in the dark conditions for 30 minutes, and then washed twice in buffer before loading to the flow cytometer.

Mitochondrial transmembrane potential

Mitochondrial transmembrane potential was analyzed using Rhodamine 123 (Rh123, Invitrogen), a lipophilic cationic fluorochrome, which accumulates inside the mitochondria in proportion to the transmembrane potential. Cell suspensions were incubated with Rh 123 (4 µl/100 ml) in HBSS for 30 min

at 37 °C, followed by washing and incubation in HBSS for 30 min. Then cells were washed twice in buffer to remove excess fluorescence probe, and were immediately evaluated using the flow cytometer.

RNA isolation and real-time quantitative polymerase chain reaction

We quantified the expression of synaptophysin and spinophilin genes in the ipsilateral side of the brain by quantitative real time polymerase chain reaction (RT-qPCR) immediately after (0 h), 3 h and 12 h after HI for all the experimental groups (n=5, with triplicates). Synaptophysin is a calcium-binding protein that lies on the membrane of presynaptic vesicles and spinophilin is a protein phosphatase 1 binding protein localized to dendritic spines. Total RNA was isolated from the brain by using RNA Mini Kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were estimated using Multi-Mode Microplate Reader (BiotekInstruments, INC Winooski, Vermont, USA). The quality and the quantity of total RNA were assessed by analysis of the ratio of A260/280 and A260/230. 1µg RNA was used as a template for cDNA synthesis using Fluidigm Reverse Transcription Master Mix (Fluidigm). RT-qPCR was performed by Fast Gene Expression Analysis using EvaGreen on the BioMark HD Nanofluidic qPCR System combined with GE 96.96 Dynamic Arrays IFC System. For mRNA detection, Prime Time qPCR predesigned primers were used (IDT Integrated DNA Technologies) (Table 1). Different reference genes were chosen for each time point between 7 housekeeping genes using Genorm and Normfinder programs, which implement an algorithm for identifying the optimal normalization gene among a set of candidates: at 0 h, HPRT and PGK1; at 3 h B2M, HPRT and PPIA; and at 12 h PGK1, PPIA and RPLPO. All samples were run in triplicate. Raw data and input data analysis was performed following the manufacturer's instructions (Fluidigm Real-Time PCR Analysis Software version 3.1.3). Relative expression levels of the genes were analyzed using the $2^{-\Delta\Delta CT}$ method in comparison to the expression of the endogenous control genes with GenEx software (MultiD).

Table1. Primers used in RT-qPCR.

GENE	GENBANK ID	PRIMER SEQUENCES
REFERENCE GENES		
PPIA	NM_017101(1)	Forward: CTGATGGCGAGCCCTTG Reverse: TCTGCTGTCTTTGGAACTTTGTC
HPRT1	XM_003752155(2)	Forward: AACAAAGTCTGGCCTGTATCC Reverse: TTCCTTGGTCAAGCAGTACAG
PGK1	NM_053291(1)	Forward: CACAGCCTCAGCATATTTCTTAC Reverse: AGATTACCTTGCCTGTTGACTT
RPL90	NM_022402(1)	Forward: GAAGCATTTTGGGTAGTCATCC Reverse: GTCCTCATTAGAGTGACATCGTC

B2M	NM_012512(1)	Forward: ATGGAGCTCTGAATCATCTGG Reverse: AGAAGATGGTGTGCTCATTGC
TBP	NM_001004198(1)	Forward: CCAGAACTGAAGATCAACGCA Reverse: TGACCTAAAGACCATTGCACT
GAPDH	NM_017008(1)	Forward: GTAACCAGGCGTCCGATAC Reverse: TCTCTGCTCCTCCCTGTTC
ANALYZED GENES		
Synaptophysin	NM 012664	Forward: GAA CAC ATG CAA GGA ACT GAG Reverse: GAA CAC GAA CCA TAA GTT GCC
Spinophilin	NM 053473	Forward: CGA TCC AAG TAT TCA GCA CCT Reverse: AGT CCT TCT CCA ACT CCA CA

Behavioral impairment evaluation

Long-term behavioral studies were performed using P90 rats: Control, HI and HI+DHA (n=14). All tests were performed by a researcher who was blind to the group to which the animals pertained.

Open field test

Motor activity was evaluated using the open-field test which consists of a rectangular container made of dark polyethylene (60×60×30 cm) to provide optimal contrast to the white rats in a dimly lit room. The base of the cage is divided by lines in central squares (4) and peripheral squares (12). Rats were individually placed in the center of the apparatus to initiate a 30 min test session, in a silent room with constant light. Each session was registered with a videocamera and directly analyzed with the SMART (Spontaneous Motor Activity Recording & Tracking) v.3.0 software system (Panlab, Barcelona, Spain). Total, central and peripheral covered distance (mm), velocity (mm/s) and the time spent in both areas were analyzed.

Hole-board test

Anxiety measured using the hole-board test, which involved using a grey iron plate covered with dark formica (62×62×36 cm) with a raised floor insert (7 cm above the floor) with 16 holes, each with a diameter of 5 cm. Each rat was placed in the center of the apparatus and left to explore the arena for 5 minutes. The frequency of and time spent head dipping into the holes was recorded as a measure of neophobia (anxiety). A head dip was scored when the head was introduced into the holes at least to the level of the eyes.

T-maze test

T-maze alternation task was used to test working memory. The experiments were performed in a T-maze constructed of wood and painted brown. The walls were 23 cm high, and the alleys were 18 cm wide. The length of the main alley was 31 cm, and the length of the side alleys was 31 cm. The side alleys were closed

off from the main alley by movable doors. A week before habituation, all animals were partially food restricted (each female received 15 g of food per day and each male received 20 g of food per day) and remained that way throughout the remaining part of the experiment. This maintained each animal above 85% of its free-feeding body weight. The T-maze was cleaned between different animals but not between different trials. The food reward was a 5 g food pellet. The full experiment consisted of three parts: habituation, training, and testing. During habituation, all animals were placed on the T-maze until they ate two pieces of food or 90 s had elapsed. This was repeated three times a day for 5 days. During training, all animals underwent six trials a day. Each trial consisted of two runs: a forced run and a free run. On the forced run, rats were forced to obtain a piece of food from one goal arm of the T-maze, with the other goal arm blocked by its door. Animals were then placed back into the start arm for a 10 s delay period. At the beginning of the free run, the rats were allowed to choose either goal arm. If the rats chose the arm opposite the one they had been forced into during the forced run, they received the food reward. If the rats chose the same arm into which they had been forced, they received no food reward. There was a 5 min inter-trial interval. The training period ended after control animals made 70% correct choices on 2 consecutive days. Rats were then tested for their performance at 10 or 40 s delay periods. Rats were given three 10 s delay and three 40 s delay trials during the day of testing. The sequence of delays and forced-run food locations (left or right) were randomized each day, with the stipulation that the same delay or the same forced-arm location could not be used for three trials in a row. Rats were then tested for their performance in the maze recording the number of correct entries. Goal entries were defined as the placing of the four paws in the arm.

Novel object recognition test

Visual episodic memory was measured by novel recognition object test (NORT), a non-rewarded paradigm test. Animals were first habituated to the experimental room for a 30 min period. On the first day, animals were habituated to the apparatus (15x28x50 cm) for a 10 min period. On the next two days, animals were allowed to freely explore two identical novel objects for a 10 min period. On the test day, one of the objects was replaced by a new different one and the animal was allowed to freely explore for 10 minutes. Exploratory behavior was scored for investigation time of each object in the test session. The discrimination index ([time in new object minus time in familiar object] / [time in new object plus time in familiar object]) was defined as the parameter for evaluation.

Axonal anterograde tracing experiments

Long-term tracing experiments were performed to evaluate the neuronal connections when animals were 100 days old (P100): Control, HI and HI+DHA ($n \geq 3$). Animals were anaesthetized with an i.m. injection of a mixture of ketamine chlorhydrate (36 mg/kg body weight, Sigma-Aldrich) and xylazine (6.2 mg/kg body weight, Rompun, Bayer, Barcelona, Spain). Once analgesia was achieved, the skin overlaying the skull was incised and a hole was drilled in the cranium following stereotaxic coordinates. For anterograde tracing experiments, biotinylated dextran amines (BDA, Sigma; 10% in 0.01 M PB, pH 7.4) were injected into the left lateral of the cortex at the level of the somatosensory barrel cortex and auditory cortex at interaural distance 5.40 mm and bregma -3.60 mm level [25]. The tracer was pressure-injected at a rate of 0.15 µl/min with a 33 G needle and syringe (nanofil syringe, World Precision Instruments, USA) connected to a microinjection unit (kds310, Kd Scientific, USA).

After surgery, the rats were kept warm with an electric blanket until they had recovered from the anaesthesia. 7 days postsurgery (P107), animals were anaesthetized as above, and perfused transcardially for 1 min with 0.1 M PBS (pH 7.4), followed by cold PFA 4% in 0.1 M PB (pH 7.4). Thereafter, the brain was removed, postfixed overnight in the same fixative, and then cryoprotected in 30% sucrose in PBS.

To reveal anterograde transport of BDA, 60-µm cryotome coronal sections were treated for 30 min with 1% H_2O_2 in PBS to inactivate endogenous peroxidase, and then thoroughly rinsed in PBS to completely eliminate the H_2O_2. Then free-floating sections were incubated overnight at 4 °C with 1:200 avidin-biotin-peroxidase (Vectastain kit, Vector Laboratories) in PBS containing 0.5% Triton X-100. The reaction product was developed in DAB (300 µL of DAB and 8.3 µL of H_2O_2 in 50 mL of 0,05 M Tris–HCl, pH 7.4). The sections were mounted on gelatin coated slides, dehydrated in ethanol, cleared in xylene, and coverslipped with DPX mounting medium (Fluka Química, Madrid, Spain). Images were taken with an Optiphot-2 Nikon microscope and a Nikon DMX 1200Fdigital camera.

Statistical analyses

Statistical analysis was performed using GraphPad Prism version 5 (Graph Pad Software, San Diego, CA, USA). Data were analyzed for the presence of outliers using Grubbs' outlier test. All data were expressed as the mean ± standard error of the mean (SEM) and were analyzed using a one-way analysis of variance

followed by Bonferroni-Dunn correction.

Results

Morphological evaluation

DHA reduced infarcted area and brain injury

Nissl staining was performed to evaluate the possible damage on tissue 7 days after hypoxic-ischemic injury (i.e. at P14). Staining of brain sections macroscopically revealed evident signs of infraction in the ipsilateral side of the hippocampus in the HI group while DHA-treated and control animals did not (Fig. 1).

Fig. 1 Representative stereomicroscopic photographs of brain sections (interaural distance 5.40 mm and bregma -3.60 mm) stained with tolonium chloride at P14. (**a**) Control group, (**b**) Hypoxic-ischemic brain with obvious damage to the hippocampus, and (**c**) DHA-treated brain similar to the control brain (n=8). Scale bar: 2.5 mm.

Brain injury was evaluated using total tissue loss volume and histopathological scoring. The area of infarction was defined as the loss of the normal cresyl violet staining pattern measured using Image J software (Fig. 2a). The HI group presented a high percentage of damage (28.7 ± 3.2%) in comparison the control group (0.9 ± 0.8%), while no statistical difference (P<0.0001) was found between animals pretreated with DHA (4.6±0.6%) and the control group. The second way to quantify the damage caused by hypoxia-ischemia was by using a semi-quantitative histopathological scoring system, modified from the one presented by Hedtjärn et al. [28] (Fig. 2b). These results revealed that animals pre-administered with DHA obtained a significantly lower score (P<0.0001) in comparison to the DHA-untreated animals.

Fig. 2 Brain injury quantification in animals exposed to hypoxia-ischemia (HI) at P7 and evaluated at P14 in the Control (n=11), HI (n=8) and HI+DHA (n=14) groups. **a) Percentage of infarcted area,** calculated from both hemispheres using the formula 100(L-R)/L, where R is right-side or ipsilateral hemisphere and L is left-side or contralateral hemisphere. b**) Histopathological score of damage in P14 rats brain (maximum score 28)**. Each bar represents the mean ± SEM (**P<0.005 or ***P<0.0001 vs. Control; $^{###}P$<0.0001 vs. HI).

DHA attenuated cell damage in the hippocampus and in the parietal cortex

Similarly, histological analysis of the degree of injury seven days after the HI insult revealed that the DHA-pretreated animals presented less cell damage in the different areas of the hippocampus and in the parietal cortex than the animals subjected to HI alone (Fig. 3).

Fig. 3 Representative microphotographs of Nissl-stained whole ipsilateral hippocampus and its different areas (CA 1, CA 2–3 and DG) and of the parietal cortex (CTX) in animals exposed to hypoxia-ischemia (HI) at P7 and evaluated at P14. Individual fields represent different experimental groups: (**a-e**) control, (**f-j**) HI and (**k-o**) HI+DHA. The hippocampus in the HI animals displayed an evident infarction, while those of the pretreated animals did not. (a, f, k): scale bar 500 µm; and (b-e, g-j and l-o): scale bar 50 µm.

Fig. 4 illustrates that DHA treatment was associated with a significantly lower histopathological score in HI vulnerable brain regions, compared with non-DHA pretreated animals, especially in the CA 1 and CA 2-3 areas of the hippocampus. In contrast, in the HI brains, injury was more severe in the CA 1 and dentate gyrus.

Fig. 4 Brain injury scores in P14 rat brains from the different groups, control (n=11), HI (n=8) and HI+DHA (n=14), expressed as the mean ± SEM. Results from the (**a**) CA 1, (**b**) CA 2-3 and (**c**) DG areas of the hippocampus and **(d)** the parietal cortex demonstrated that DHA protected the brain from subsequent H-I injury, especially in the CA 1 and CA 2-3 areas. (****P*<0,0001 vs. Control; $^{###}P$<0.0001 vs. HI).

Myelination was maintained by DHA treatment

MBP immunostaining demonstrated differences among groups at the level of the external capsule (Fig. 5a-c) and mid-striatum (Fig. 5d-f), evaluated at P14. We found that the animals that underwent HI presented a reduction in the expression of MBP with respect to controls, whereas this loss was absent in DHA pre-treated animals.

Fig. 5 Representative light microphotographs of myelin basic protein (MBP)-stained brain sections (a-c) in the external capsule and (d-f) in the striatum of different groups evaluated at P14: (**a, d**) control, (**b, e**) HI and (**d, g**) HI+DHA (scale bar: 40 µm).

White matter integrity was analyzed by measuring the density of MBP immunostaining in both these areas of the brain (Fig. 6), which corroborated what was apparent at a microscopical level. An important loss of ipsilateral MBP immunostaining ($P<0.0005$) was measured in the HI group in both areas, but particularly in the striatum (Fig. 6b) when compared with control. In contrast, we found that pups pretreated with DHA presented similar (I:C) MBP ratios to those exhibited by the control group. Thus, DHA treatment resulted in the maintenance of MBP levels in the white matter of the ipsilateral hemisphere in both anatomical regions.

Fig. 6: Histograms illustrating the measurement of the density of myelin basic protein (MBP) immunostaining (a) in the external capsule and (b) in the striatum of the Control (n=5), HI (n=14) and HI+DHA (n=11) groups. The extent of tissue injury, expressed as a ratio of left-to-right hemispheric MBP immunostaining, is represented. Each bar represents the mean ± SEM (*$P<0.05$ or ***$P<0.0001$ vs. Control; $^{\#\#\#}P<0.0001$ vs. HI).

The effects of DHA after HI on gliosis

GFAP was used as a marker of astrocytes, which can be seen as star shaped cells under the microscope (Fig. 7). Remarkable astroglial reactivity was observed in the HI group in comparison with control animals in the ipsilateral parietal cortex and CA 1 area of the hippocampus. In contrast, in the HI+DHA group there was a reduction in GFAP staining compared to the HI group, indicating that DHA minimized the astroglial reactive response in these areas.

Fig. 7 Representative confocal photomicrographs of glial fibrillary acidic protein (GFAP)-positive cells in neonatal rat brain at postnatal day 14 (**a-c**) in the parietal cortex and (**d-f**) in the CA 1 area of the hippocampus, of the (**a, d**) control, (**b, e**) HI, and (**c, f**) HI+DHA groups. These areas of animals with

HI injury demonstrated intense GFAP staining, which was substantially reduced in the DHA-pretreated group. Scale bar: 40 µm.

DHA reduced microglial activation

Microglia were stained using the macrophage marker Iba-1 (Fig. 8). An evident increase in the number of Iba-1-postive activated microglial cells could be seen in the dentate gyrus and CA 1 areas of the hippocampus of HI animals with respect to controls. In contrast, low levels of Iba-1 expression were observed in the case of the DHA pretreated group, indicating that diminished microglial activation was associated with DHA pretreatment.

Fig. 8 Representative confocal microphotographs of brain sections stained with ionized calcium binding adaptor molecule-1 (Iba-1) and counterstained with DAPI (red), (**a-c**) in the dentate gyrus and (**d-f**) in the CA 1 areas of the hippocampus, of the (**a, d**) control, (**b, e**) HI, and (**c, f**) HI+DHA groups. On postnatal day 14, Iba-1 (green) immunoreactivity was substantially reduced in the DHA pretreated group in comparison to the HI group. Scale bar: 40 µm**.**

Evaluation of mitochondrial state

DHA pretreatment preserved mitochondrial inner membrane integrity

At the earliest studied states, i.e. 0 h and 3 h post infarction, no significant differences were found among all groups ($P<0.005$), indicating that the integrity of the mitochondrial inner membrane integrity was unaffected by the HI (Fig.9a, b). In contrast, at 12 h, the HI group exhibited a significant diminishment (72.3±12.0%; $P<0.05$) in the percentage of NAO positive cells compared with that of the control group (98.0±0.6%). Animals pretreated with DHA maintained mitochondrial integrity (98.29±0.84) and showed similar values with respect to the control group (Fig. 9c). In regards to the relative values of fluorescence intensity for NAO, a reduction was observed in the HI group (57.53±5.24) at 0 h in comparison with the control group ($P<0.05$). In contrast, the HI+DHA group was able to prevent this diminishment (Fig. 9d). At 3 h, we did not found any statistically significant difference among the experimental groups (Fig. 9e). Similarly at 12 h no difference was observed between HI and control groups, but the HI+DHA group presented an evident increase in the relative values of NAO fluorescence intensity (236.9±61.63) respect to

the HI (71.31±6.18) and control groups (P<0.05) (Fig. 9f). The data obtained from both the percentage of positive cells with NAO and the relative fluorescence intensity in acutely isolated cells suggest that DHA protected mitochondrial inner membrane integrity.

Fig. 9 Evaluation of the mitochondrial inner membrane integrity using nonyl acridine orange (NAO) staining of acutely isolated cells in suspension. (a-c)Percentage of NAO-positive cells and (d-f) relative fluorescence intensity of cells with the NAO *in vivo* marker at different time points after hypoxia-ischemia: **(a, d) 0 h, (b, e) 3 h and (c, f) 12 h**, in control, HI and HI+DHA groups (n≥5). Each bar represents the mean ± SEM (*****P<0.05 vs. Control; $^{\#}P$<0.05 or $^{\#\#}P$<0.005 vs. HI).

DHA maintained mitochondrial membrane potential

In all the studied time points, the HI group presented widespread reduction in the percentage of Rh 123 positive cell respect to the control group, whereas no significant differences were found between HI+DHA and control groups (Fig. 10a-c). Regarding the relatives values of fluorescence intensity of Rh 123, at 0 h we found that the animals suffering an H/I injury showed a diminishment (56.33±4.83), while animals pretreated with DHA (81.85±13.5) demonstrated similar values to the control group (P<0.05) (Fig. 10d). On the contrary, at 3 h the HI group treated with DHA presented an augmentation of the relative fluorescence intensity (139.3±2.79) when comparing with both control and HI groups, but no significant difference was observed between control and HI groups (P<0.05) (Fig. 10e). At 12 h no statistically significant difference was appreciate among the groups (Fig. 10f). The results indicate that DHA maintained mitochondrial transmembrane potential in all of the studied time points.

Fig. 10 Effect of brain hypoxia-ischemia on the mitochondrial transmembrane potential of acutely isolated brain cells evaluated by Rhodamine 123. (a-c) Percentage of cells labeled with the *in vivo* marker Rh 123 and (d-f) relative fluorescence intensity of cells exhibiting Rh 123 at different time points after hypoxia-ischemia: **(a, d) 0 h, (b, e) 3 h and (c, f) 12 h**, in control, HI and HI+DHA groups (n≥5). Each bar represents the mean ± SEM ($^{*}P$<0.05, $^{**}P$<0.005 or $^{***}P$<0.0001 vs. Control; $^{\#\#}P$<0.005 or $^{\#\#\#}P$<0.0001 vs. HI).

Synaptophysin and spinophilin expression

Changes in the expression of synaptophysin gene after the HI injury

Synapthophysin was down-regulated at 0 h in the animals that underwent the hypoxic-ischemic injury [HI: fold-change -0.73±0.13, HI+DHA: fold-change -1.05±0.14; $P<0.05$) (Fig. 11a) comparing with the control group. On the contrary, at 3 h there was not any significant difference between the HI and the control group, but the DHA-treated group revealed an up-regulation (fold-change 0.58±0.11; $P<0.05$) (Fig. 11b). However, 12 h after the hypoxic-ischemic injury, the expression of synapthophysin was down-regulated again in the HI group (fold-change -0.52±0.06; $P<0.05$) in comparison to the control group, whereas animals treated with DHA underwent an increase (fold-change 0.06±0.14; $P<0.05$) in the expression respect to the HI group, showing similar values to the control group (Fig. 11c).

Fig. 11 Synapthophysin expression in the ipsilateral side of the brain of postnatal rats was determined by RT-qPCR at different time points after hypoxia-ischemia, **(a) 0 h, (b) 3 h and (c) 12 h**, in control, HI and HI animals pretreated with DHA (n=5, with triplicates). Fold changes illustrated in the graphs were quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SEM ($^{*}P<0.05$, $^{**}P<0.0005$ or $^{***}P<0.0001$ vs. Control; $^{\#\#}P<0.0005$ vs. HI).

Changes in the expression of spinophilin gene after the HI injury

At 0 h, animals from HI group demonstrated a significant reduction of spinophilin mRNA quantity (fold-change -1.06±0.36; $P<0.05$) respect to the control group, while animals pretreated with DHA did not (Fig. 12a). At 3 h, the DHA group showed an augmentation of the quantity (fold-change 0.95; $P<0.05$) respect to the control (Fig. 12b). On the contrary, at 12 h the expression of spinophilin was unaffected in any experimental group (Fig. 12c).

Fig. 12 Spinophilin expression in the ipsilateral side of the brain of postnatal rats was determined by RT-qPCR at different time points after hypoxia-ischemia, **(a) 0 h, (b) 3 h and (c) 12 h**, in control, HI and HI animals pretreated with DHA (n=5, with triplicates). Fold changes illustrated in the graphs were quantified by normalization to the reference genes specific for each time point. Each bar represents the

mean ± SEM (*P<0.05 vs. Control).

Assessment of behavioral parameters

Four behavioral tests were carried out on postnatal day 90 in control, HI and treated rats in order to test if administration of DHA could improve the behavioral deficits produced by the asphyctic injury.

Motor activity was not affected by the HI injury

Animals that underwent hypoxic-ischemic injury did no present any significant difference when comparing with control group in the motor activity analyzed by the open field test in any of the evaluated parameters, such as the percentage time in the periphery (Fig. 13a), percentage time in the center (Fig. 13b), distance travelled in the periphery (Fig. 13c), distance travelled in the center (Fig. 13d), speed in the periphery (Fig. 13e) and speed in the center (Fig. 13f). Likewise, no significant differences were found between control and HI animals pretreated with DHA. These results demonstrated that hypoxic-ischemic injury did not alter motor activity.

Fig. 13 Motor activity evaluation using the open field test at P90 in control, HI and HI+DHA groups (n=14). (**a**) %time in the periphery, (**b**) %time in the center, (**c**) distance travelled in the periphery, (**d**) distance travelled in the center, (**e**) speed in the periphery and (**f**) speed were evaluated in the center in the open field. Each bar represents the mean ± SEM.

Anxiety was reduced by the DHA

There was a significant main effect of head dipping frequency in the HI group, which had lower frequency (37) than the control group (20), while no significative difference could be observed between HI+DHA (32) and control groups (Fig. 14a). These results suggest that the docosahexaenoic acid is able to reduce the long-lasting anxiety and neophobia caused by the hypoxic-ischemic brain injury. In regards to the time spent head-dipping into the holes, no differences were found among the experimental groups (Fig. 14b), however, the HI group spent lower duration of head dipping than the control and HI+DHA groups, but it was not statistically significant.

Fig. 14 Evaluation of anxiety and neophobia using the hole-board test. (**a**) The frequency and (**b**) the time spent head-dipping into the holes were recorded for the control, HI and HI+DHA groups (n=14). Values are expressed as mean ± SEM (***P*<0.005 vs. Control).

Spatial memory outcomes were ameliorated by the DHA

In order to evaluate spatial reference and working memory, control, HI and pretreated animals were subjected to the T-maze test at two delay intervals. While at a 10 s delay interval no differences were observed among groups (Fig. 15a), at a 40s delay interval the HI group presented significantly fewer number of correct choices (26.00 ±5.14) comparing with the control (88.21± 14.38). On the contrary, rats treated with DHA made similar number of correct choices (66.71± 7.02) to the control group (*P*<0.0001) (Fig. 15b). The results point out that hypoxia-ischemia leads to spatial working memory outcomes and that these can be avoided by the DHA.

Fig. 15 Assessment of spatial working memory was performed using the T-maze on P90 rats in control, HI and HI+DHA groups (n=14). Animals were tested at two delay intervals: 10 s and 40 s. (**a**) No significant differences were found at 10 s delay. (**b**) At 40s delay, HI animals made significantly fewer correct choices compared with control animals, whereas DHA reverted the HI-impaired delayed alternation of memory performance. Values are expressed as mean ± SEM (**P*<0.05 or ****P*<0.0001 vs. Control; $^{\#\#\#}$*P*<0.0001 vs. HI).

DHA preserved recognition memory after the brain damage

Exploratory behavior and long-lasting non-spatial recognition memory were evaluated by novel object recognition test (Fig. 16). The discrimination index indicated that the animals that underwent hypoxia-ischemia presented a significant diminishment comparing with the control ones, which means that they spent less time in the novel object than in the familiar one. In contrast, the treated rats spent more time exploring the new one, recovering the discrimination index to control values (*P*<0.005).

Fig. 16 Exploratory behavior was scored using the novel object recognition test on P90 rats. In a 10 min test session, the discrimination index ([time in new object minus time in familiar object] / [time in new object plus time in familiar object]) was defined as the parameter for evaluation. The HI group demonstrated a robust decrease in discrimination index with respect to control, whereas DHA pretreatment fully reversed this reduction. Each bar represents the mean ± SEM ($^{**}P<0.005$ vs. Control; $^{\#\#}P<0.005$ vs. HI).

DHA prevented the changes in anterograde axonal connections caused by the HI injury

Seven day later (P107) changes in the axonal connections of corticofugal neurons following neonatal hypoxic-ischemic injury were detected in the HI rats in the corpus callosum, there were fewer labeling fibers. In contrast, these changes were reverted with DHA pretreatment, demonstrating similar patterns to control rats (Fig. 17a, b, c). It was similar in the thalamus of the ipsilateral side (Fig. 17g, h, i) and in the cortex of the contralateral side (Fig. 17d, e, f) of the brain, where the animals that underwent the injury showed fewer immunostained neurons in comparison to the control group, while animals that received DHA presented almost the same pattern of staining of the control animals.

Fig. 17 Representation in coronal slices of the extension of immunostained neurons in the corpus callosum (a, b, c), in the ipsilateral thalamus (d, e, f) and in the contralateral cortex (g, h, i), after anterograde tracer injection of biotinylated dextran amines (BDA) into the ipsilateral cortex on P107 rats in the control (a, d, g), HI (b, e, h) and HI+DHA (c, f, i) groups. HI rats presented fewer labeled fibers in these three regions, whereas DHA-treated rats exhibited staining patterns which were similar to control ones. Scale bar: 40 μm.

Discussion

Taken as a whole, the present prospective study has revealed for the first time that docosahexaenoic acid preadministration can neuroprotect long-term cognitive functions related to memory and learning processes, as well as the integrity of corticofugal connections in an experimental model of neonatal hypoxic-ischemic encephalopathy. DHA therapy resulted in improved behavioral function which was associated with the prevention of loss of brain volume after injury.

Histological examination seven days after the injury indicates that DHA administration prior to HI is able to reduce infarcted area, as revealed by Nissl staining, corroborating the findings of Berman et al. [24], Williams et al., which administrated two doses of n-3 TG (containing both DHA and eicosapentaenoic acid [EPA]) [29] and Zhang et al., who administrated omega-3 polyunsaturated fatty acid supplementation (DHA+ EPA) to pregnant female rats [30]. Moreover, it reduced cell loss and the number of swollen and damaged neurons caused by hypoxia-ischemia. DHA protected not only the neurons, but also the rest of the cell populations of the CNS, since myelin was preserved and both the astroglial reactive response and microglial activation were diminished. HI leads to myelination deficits causing white matter lesions and damage to gray matter oligodendrocyte progenitors [31-32]. Reduced MBP staining, considered to be a hallmark of diffuse white matter damage in fetal rodents [33] and preterm infants [34], was observed in subcortical white matter at the level of the external capsule and striatum in the HI group. In contrast, a recovery of that MBP staining was found in the HI+DHA group, corroborating earlier findings by our group [35], who found similar recovery at the level of the inferior colliculus.

DHA reduced GFAP expression, indicative of astrocyte protection, in contrast to the findings of Belayed et al. [36] and Eady et al. [37], who demonstrated an increase in the number of GFAP-positive cells with DHA treatment after acute ischemic stroke in adult rats. Elevated GFAP levels are indicative of increased glial reactivity, and this increased expression is considered to be a specific biomarker of the severity of neonatal HI encephalopathy [38]. In the same way, it is well established that HI-induced inflammation contributes to the development of injury in the newborn brain [39-41]. Even though quick microglial activation has been recognized to be beneficial for brain recovery, prolonged overstimulation of microglia may be harmful [42-43]. Our HI group showed an increased expression of Iba-1 in the hippocampus, in keeping with previous findings [39, 44-46]. In contrast, pretreatment with DHA robustly reduced the expression of Iba-1, as reported by Zhang et al., who found that dietary omega-3 polyunsaturated fatty acid supplementation (DHA+ EPA, 15 mg in every gram of regular diet) to pregnant female rats had the same effect [43]. These

results thus clearly indicated that the mitigation of microglial activation is clearly associated with neuroprotection and further corroborate the anti-inflammatory properties of the omega-3 fatty acid and especially of the DHA, consonantly with Belayed et al. [36] and Zhang et al. [30].

The state of mitochondria is an important factor that determines whether injured neurons survive [19, 44-46]. Here, we report for the first time the prevention of the mitochondrial damage by DHA treatment at the level of the middle hippocampus. On the one hand, DHA conserves the number of cells with intact mitochondrial inner membrane and increases the quantity of cardiolipin in those cells, especially 12 h after the HI event. On the other hand, whereas HI animals showed a decrease in the number of Rh 123 positive cells in all studied time points, DHA pretreatment was found to maintain the percentage of cells with intact transmembrane potential, since the corresponding Rh 123 fluorescence intensity was similar to that measured in controls, indicating that DHA also protects mitochondrial membrane potential. These findings are consistent with previous reports by our group [35] and the recently published work of Mayurasakorn et al., who found that the post-administration of triglyceride emulsions of DHA preserved Ca^{2+} buffering capacity in cerebral mitochondria [49].

Instantly after HI brain damage, changes in the synaptic functions occur in the hippocampal pyramidal cells of neonatal rats [50]; synaptic transmission is rapidly attenuated by HI and after reperfusion it briefly returns only to decrease as the secondary phase of energy depletion occurs [51]. We evaluated the synapses as an indicator of neuronal plasticity by analyzing the expression of synaptophysin and spinophilin mRNA, and found that after bilateral common carotid artery occlusion there was a diminishment in the expression of both genes. Whereas synaptophysin takes part in vesicle delivery, in the development of nerve synapses and in the adjustment of the plasticity of nerve synapses [50, 52], spinophilin modulates both glutamatergic synaptic transmission and dendritic morphology [53-54]. Thus, our findings could be taken to mean that synaptic transmission is reduced just after the brain injury in the Rice-Vannucci model. During development, hypoxia can affect neurite outgrowth and synaptogenesis with subsequent long-term impairments in cognitive functions [55-56]. However, with DHA pretreatment, we did not observe this diminishment, indicating that DHA may also confer neuroprotection at the level of synapses. This is not surprising since DHA is known to be essential for the proper brain functioning, playing a fundamental role in the maintenance of membrane ionic permeability and in the function of the transmembrane receptors which support synaptic transmission and cognitive functions [7, 11-13].

Numerous clinical trials have demonstrated how important the omega-3 polyunsaturated acids are in the pregnancy and lactation. High DHA levels in the first few weeks of life are associated with decreased intraventricular hemorrhage, improved microstructural brain development, and improved outcomes in preterm born children [57]. In contrast, an inadequate dietary intake of omega-3 fatty acids reduces DHA and augments omega-6 fatty acids in the brain; this decrease of DHA in the developing brain leads to impairments in neurogenesis, neurotransmitter metabolism, and learning and visual function in animals [58-60]. We aimed to evaluate the neuroprotective effect of DHA when used as pretreatment in this experimental hypoxic-ischemic model due to the numerous positive results that DHA have previously demonstrated both in experimental ischemic models in adult and in neonate rodents. The literature concerning DHA therapy against cerebral HI injury is relatively heterogeneous regarding dosage. Encouraged by the article of Berman et al. [24] we made a histological study comparing two concentrations (1 mg/kg vs. 3 mg/kg). Then after not having obtained any statistically significant difference between them in the neuropathological evaluation (data not showed), we decided to use 1 mg/kg of DHA.

The behavioral consequences of HI encephalopathy in children often include functional and intellectual deficits, and to reveal the true functional disability it is important to do long-term follow-up studies [56]. Likewise, when exploring the potential neuroprotective effects of any treatment, it is essential to confirm that the apparent benefits are reflected in a long-term neurological status [61]. Thus, we performed different behavioral tests to evaluate long-term locomotor activity, anxiety, and spatial and working memory. In line with previous results, the present study revealed no influence of brain injury on gross motor activity, measured in an automated open field apparatus. In general, the HI rats moved like control ones and they did not present abnormalities in locomotion or posture [26, 62-63], reflecting the integrity of the cerebellum, which subserves such functions. In contrast, anxiety, measured as an increased frequency of head-dipping in the hole-board test, was observed in the HI group, indicating that HI is associated with long-term anxiogenic effects. DHA-treated rats manifested a frequency of head-dipping similar to that of controls, consistent with several reports of the anxiolytic benefits of omega-3 fatty acids, even in humans [64-67]. Impairments in reference and working memory after neonatal HI were detected in the T-maze performance, as previously reported [21, 62, 68-74]. The results were progressively worse at increasing delays, while they were stable with DHA pretreatment, corroborating the finding of Mucci et al. [75], who have recently demonstrated how flaxseed consumption, rich in DHA's precursor α-linolenic acid (ALA), during gestation and lactation was able to improve spatial memory, and the works of Zhang et al. [30, 43] and Mayurasakorn

et al. [49]. The novel object recognition test assesses deficits in non-spatial working memory, especially visual episodic memory, and is based on the innate preference of rats to examine novel objects rather than familiar ones [61, 76-77]. The results reported here support the idea that the pretreatment with DHA can be therapeutically efficacious and lead to ameliorated behavioral impairments, and indicate that neuroprotection against neonatal HI can persist, even into adulthood. This protection of cognitive function was correlated with a significant reduction in morphological brain damage in the cerebral cortex and hippocampus, brain areas known to be essential for the acquisition and retention of spatial memory tasks. In fact, the cognitive dysfunctions induced by HI may result from abnormal communication within prefrontal-hippocampal networks, because synchrony and directed interactions between the prefrontal cortex and hippocampus account for mnemonic and executive performance [78]. In the same way, DHA deficiency during development is associated with impairment in learning and memory, suggesting an important role for DHA in neuronal development [79].

As we have already mentioned, the prefrontal cortex is involved in the processing of working memory [21] and this area is damaged by the HI. For this reason, we evaluated alterations in neuronal connections to this cortex following HI and found DHA to moderately protect against changes caused by HI injury. Hence, anterograde tracing experiments revealed that DHA treatment partially avoided the alterations observed in the immunostained pattern of the contralateral cortex, the ipsilateral thalamus and the corpus callosum, after HI, suggesting that at least in part DHA can improve axonal remodeling following HI insults.

It is widely recognized that the young nervous system possesses a greater capacity to recover from an injury than the adult nervous system. This potential has been attributed to the greater plasticity that exists in the neocortex of young animals as opposed to adults [80], and it seems that DHA may help by increasing this plasticity. Recovery of function has been related to the survival of neurons that would otherwise have degenerated and/or to the synaptic reorganization at local or regional levels. It could be suggest that memory recovery after DHA treatment may be mediated by prevention of alterations in corticofugal neuronal connections and, obviously, by the reduction of cerebral damage at the level of the hippocampus and parietal cortex. In fact, it is already established that the exposure to n-3 fatty acids enhances adult hippocampal neurogenesis associated with cognitive and behavioral processes, promotes synaptic plasticity by increasing long-term potentiation and modulates synaptic protein expression to stimulate the dendritic arborization and new spines formation [81]. What is more, the subchronic ALA treatment have demonstrated its capacity to induce neurogenesis in the hippocampus, especially in the subgranular zone of

the dentate gyrus, after middle cerebral artery occlusion model in adults; and this increase in the number of proliferating immature neurons might be related with motor and cognitive functional recovery [82]. In this sense, one of our next goals would be to prove that the improvement of the behavioral deficits produced by the HI injury was also supported by an increased in neurogenesis associated to the DHA treatment. And lastly, as DHA is a promising candidate to use as an add-on treatment of the standard therapy of hypothermia for reducing the brain damage induced by HI, due to its well-established safety record and its ability to readily cross the blood-brain barrier, we would like to evaluate the possible neuroprotective effect of the combination of DHA pretreatment with delayed hypothermia.

Conclusion

Our results show that DHA exerts potent and prolonged neuroprotective effects in this experimental model of perinatal hypoxia-ischemia in rats. At medium term, DHA administration prior to HI is able to ameliorate tissue loss and cell damage and to preserve the myelin, while diminishing the astroglial reactive response, as well as microglial activation caused by bilateral common carotid artery occlusion, and in the short term, DHA can maintain mitochondrial inner membrane integrity and transmembrane potential, as well as the synaptic function. Finally, our results provide novel evidence for the protective capacity of DHA to improve behavioral outcomes by preventing the loss of brain volume and by partially avoiding alterations in anterograde neuronal connections to the cortex. The potential of DHA as effective therapeutic candidate for reducing ischemia-induced neurodegeneration in the neonate continues to hold promise.

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