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Maternal DHA supplementation protects rat offspring against impairment of learning and memory following prenatal exposure to valproic acid☆

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Abstract

Docosahexaenoic acid (22:6n-3; DHA) is known to play a critical role in postnatal brain development. However, there have been no studies investigating the preventive effect of DHA on prenatal valproic acid (VPA)-induced behavioral and molecular alterations in offspring. The present study was to evaluate the neuroprotective effects in offspring using maternal feeding of DHA to rats exposed to VPA in pregnancy. In the present study, rats were exposed to VPA on day 12.5 of pregnancy; DHA was administered at the dosages of 100, 300 and 500 mg/kg/day for 3 weeks from day 1 to 21 of pregnancy. The results showed that maternal feeding of DHA to the prenatal exposed to VPA (1) prevented VPA-induced learning and memory impairment but did not change social-related behavior, (2) increased total DHA content in offspring plasma and hippocampus, (3) rescued VPA-induced neuronal loss and apoptosis of pyramidal cells in hippocampal CA1, (4) influenced the content of malondialdehyde and glutathione and the activities of superoxide dismutase and glutathione in the hippocampus, (5) altered levels of apoptosis-related proteins (Bcl-2, Bax and caspase-3) and inhibited the activity of caspase-3 in offspring hippocampus and (6) enhanced relative levels of p-CaMKII and p-CREB proteins in the hippocampus. These findings suggest that maternal feeding with DHA may prevent prenatal VPA-induced impairment of learning and memory, normalize several different molecules associated with oxidative stress and apoptosis in the hippocampus of offspring, and exert preventive effects on prenatal VPA-induced brain dysfunction.

Keywords: Docosahexaenoic acid (DHA); Maternal feeding; Learning; Memory; Valproic acid (VPA)

1. Introduction

Prenatal exposure to valproic acid (VPA) causes widespread alterations in brain morphology, behavior and cognitive ability and increased risk of autism spectrum disorder (ASD) in offspring. Prenatal exposure to VPA in pregnant rats is a valid model of autism with neurobiological and behavioral consequences [1–3]. Increasing evidence has shown that the deficits of spatial learning and memory are severe consequences of ASD [3,4]. ASD is a developmental disorder that displays common behavioral symptoms, including pervasive impairments in social interactions, and is accompanied by reduced global cognitive function scores [5]. Although detailed mechanisms underlying the condition have not been elucidated, there is strong evidence that oxidative stress [6] and aberrant apoptotic processes [7] play important roles in abnormal hippocampus function, the loss of neurons and memory impairment in ASD following exposure to toxins.

Docosahexaenoic acid (DHA, 22:6n-3) is the most prevalent polyunsaturated fatty acid in the central nervous system. The most important role of DHA is to promote brain development [8–10], including neuronal differentiation [10], neurite growth [11], synapse formation and photo receptor biogenesis [8]. A number of studies have indicated that DHA supplementation improves cognition [12] and enhances memory [13]. It also has a role in inhibition of neuronal apoptosis [14] and in lipid signaling in defense against oxidative stress [15]. Considering the high sensitivity of DHA to reactive oxygen species (ROS) and antiapoptosis, we hypothesized that high levels of DHA should be more resistant to ROS attack and antiapoptosis. In our previous study [3], we found that DHA supplementation in the VPA-induced male offspring rats ameliorated learning and memory impairments in this autism animal model.

During the third trimester of gestation, through the placenta, DHA is preferentially transported from maternal resources to infant circulation;

☆ Conflict of interest: The authors declare that they have no conflicts of interest. All procedures involving animals were approved by the Animal Care Committee and Ethics Committee of Harbin Medical University.

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it rapidly accumulates in the fetal brain and plays a very important role in the first period of brain development [9]. Research has shown that, compared with age- and sex-matched normal children, infants with ASD have a significantly higher incidence of learning disabilities and memory impairment as well as having lower DHA levels in their serum [16]. Experimental prenatal DHA deficiency in rodents causes neuronal loss in the hippocampus and impairment of hippocampus-dependent spatial learning and memory [17]. However, no studies have been conducted to study the effects of maternal DHA feeding on prenatal exposure to VPA which induces autism-like rat offspring, and it is not clear whether DHA supplementation during pregnancy will play preventive neuroprotection of learning and memory ability in the autism animal model.

In the present study, we hypothesized that DHA may protect against brain impairment and improve learning and memory functions by modulating antioxidant and antiapoptosis mechanisms. Therefore, we examined the effects of maternal DHA feeding on prenatal-VPA-induced cognitive impairment in male offspring and also investigated the possible mechanisms involving the reduction of oxidative damage and apoptosis in the hippocampus of prenatally VPA rats.

2. Materials and methods

2.1. Animals and drug administration

Wistar male and female rats were purchased from a commercial breeder (YISI, Changchun, China). The study protocol was approved by the Institutional Animal Care and Use Committee, Daqing Campus of Harbin Medical University. Female rats that weighed 240–280 g and male rats that weighed 280–320 g were used. Rat feeding conditions and determination of first day of pregnancy (E0) were the same as those previously described by our group [3]. On E0, each pregnant rat was housed separately and then divided into the following groups: control (same volume of sunflower oil, $n=12$), VPA (same volume of sunflower oil, $n=12$) and three DHAs (Zhejiang Hisun Pharmaceutical Co., Ltd., China). TriDHA oil was produced by Schizochytrium sp. treatment groups (100, 300 and 500 mg/kg/day DHA supplementation, which were denoted as low, middle and high groups, $n=12$, respectively). Each group was given DHA or sunflower oil *via* oral gavage for 3 weeks.

Each group of pregnant rats, except the control group, was given a single intraperitoneal injection of 600 mg/kg sodium VPA (Sigma, St. Louis, MO, USA) on E12.5. Control females were injected with the same amount of physiological saline at the same time [18]. Females were allowed to raise their own litters. The offspring of all animals that were exposed to VPA during gestation developed a characteristic "kink" in the tail [6], and they were easily distinguishable from the aged-matched control group's offspring. The offspring were weaned on postnatal day (PND) 21, and the following experiments were conducted on the male offspring. Male offspring rats were used for each group as follows: control group ($n=39$), VPA-only group ($n=36$), low-dose DHA treatment group ($n=36$), middle-dose DHA treatment group ($n=38$) and high-dose DHA treatment group ($n=37$). The same group of male offspring from different litter was distinguished by a different marker with trinitrophenol. During PND 35 to PND 40, each group of male offspring rats from a different dam was randomly selected for learning and memory testing by the Morris water maze and for sociability using the three-chamber test. The remaining untested male offspring rats were sacrificed for other assays on PND 40.

2.2. Total DHA content analyses in offspring plasma and hippocampus

Ultraperformance liquid chromatography (UPLC) analysis was performed to detect the DHA level of plasma and the hippocampus in offspring rats of different groups using the same method described previously [3,19]. Hippocampal tissue (wet weight 0.03 g) and plasma (200 μl) of the offspring were weighed, and 600 μl or 400 μl ethanol was added, respectively. Internal standards (prostaglandin B1 in 400 μl of 100 pmol/ml ethanol solution), BHT (1 μl, 0.5% w/v ethanol solution as an antioxidant) and acetic acid (2 μl, 88% v/v) were added into the samples. Samples were put on ice for 1 h to extract the arachidonic acid metabolites followed by centrifugation at 150,000 rpm at 4°C for 20 min. The supernatant was transferred to another fresh tube and diluted by 3 ml of distilled water. The solution was applied to a Strata-X polymeric SPE column (200 mg/6 ml), which was preconditioned with 100% ethanol and 25% ethanol. Eicosanoids were eluted from the column with ethyl acetate that contained 0.0002% BHT 5 ml before the ethyl acetate layer was dried by N_2 flushing on an ice bath. The dried sample was reconstituted in 0.05% acetic acid (ethanol solution, 2000 μl) as the test solution. Water Acquity TM UPLC system was used to perform the UPLC analysis. Five-microliter samples were injected into the sample injector, which was stored at 4°C throughout the analysis. Pure water was the mobile phase B. The mobile phase A was acetonitrile, and followed rate was 0.20 ml/min. The mobile phase gradient ran from 35% A to 90% A over 7 min, returning to 35% A over 1.5 min, and was carried out in 35% A for 1.5 min for reequilibration.

2.3. Behavioral tests

2.3.1. Morris water maze

Spatial learning and memory of rats were assessed by the Morris water maze. The method was performed as described previously [3]. Briefly, rats were trained five times to find the escape platform which was hidden 1.5 cm underneath the water surface. The following five times were the test results. From the sixth time to the ninth time, the escape latency (time to reach the platform) was considered to be an index of performance in this task. For rats that did not reach the platform, the latency value was equal to 60 s. At the last time, the platform was removed from the tank. The number of times that the animal passed through the circular area (diameter, 10 cm), which formerly contained the platform during acquisition, was thought to be a measure of spatial memory. The probe trial was occurred over 60 s.

2.3.2. Three-chamber sociability test

Sociability test was conducted in an automated three-chambered apparatus using methods described earlier [20]. The apparatus was made of clear polycarbonate (60 cm long×40 cm wide×20 cm high). According to the length, the chamber was separated into three parts by homogeneous partition; a switch gate (5 cm wide×7 cm high) of each partition was placed in the lower part of the central region and, through it, allowed access to the different chambers. A top-mounted CCTV camera (Security Cameras Direct, Luling, TX, USA) was placed over the boxes, and time spent in each chamber, as well as the time spent exploring the stranger rat or an object in the chamber, was automatically recorded by it and calculated by the automated software. The object was an empty wire cage (5.5-cm radius and 20-cm height) used to enclose the stranger rat. Chambers were cleaned up with 70% ethanol and water between tests. Animals utilized as "strangers" were from different litters and had no previous contact with the test rats. When the experiments were to start, rats were allowed to spend 10 min in the central chamber, and then the strange rat was introduced into one of the side chambers. The experiment was performed for up to 10 min, with the strange rat and an object on each side.

2.4. Histological tests

2.4.1. Nissl staining

After rinsing with deionized water (3 min), sections were plunged into 0.1% crystal violet for 15 min at 37°C and then rinsed with deionized water again. Then, sections were dehydrated. Sections were subsequently cover slipped with neutral balsam and viewed under a light microscope. Six brain sections were chosen from each animal and processed for counting.

2.4.2. NeuN immunohistochemistry

NeuN immunohistochemistry was conducted on 5-μm-thick hippocampal coronal sections. After washing in phosphate-buffered saline (PBS) (3×5 min), tissue sections were sequentially processed with 0.3% hydrogen peroxide for 10 min and then rinsed with PBS. Then, sections were incubated with 10% normal goat plasma for 15 min at room temperature and sequentially exposed to mouse anti-NeuN (1:50, Chemicon, CA, USA) overnight at 4 °C. After rinsing, sections were incubated with biotinylated goat–mouse IgG for 15 min at 37°C. After further washing in PBS, sections were exposed to streptavidin peroxidase (Vector, Burlingame, CA, USA) for 15 min at 37°C. Sections were rinsed in PBS (3×5 min) and then exposed to 3.3-diaminobenzidine (DAB) for approximately 5 min. They were later put in the water to stop the DAB reaction. All subsequent performances were incubated as described above, and no positive immunoreaction was observed.

2.5. Measurement of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GSH-PX)

The amount of MDA and the activities of SOD, GSH and GSH-PX were measured in accordance with the manufacturer's instructions in different commercially available assay kits (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China). The concentration of MDA was determined by measuring thiobarbituric-acid-reacting substances at a wavelength of 532 nm. The level of MDA was expressed per mg protein nmol MDA. SOD activity was examined in hippocampal homogenate by calculating the rate of inhibition of nucleotide oxidation. Results were identified as U/mg protein. The GSH concentration and GSH-PX activity were assayed by quantifying the rate of oxidation of reduced glutathione to the oxidized glutathione by H_2O_2. Protein content depended upon the mean of Coomassie brilliant blue using bovine plasma albumin as the standard.

2.6. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from hippocampal tissue using Trizol reagent (Invitrogen Technology) according to the manufacturer's protocol. The concentration of RNA was quantified from the optical density that was measured at 260 nm by ultraviolet spectrophotometry. Equal amounts of RNA (500 ng) from each sample were reverse transcribed in a volume of 10 μl to produce cDNA. GAPDH was used as an endogenous internal standard control. SYBR Green I-based detection was conducted on a real-time PCR instrument (ABI PRISM 7300) with thermal cycler data being expressed in a ratio: the relative quantity of Bcl-2, caspase-3 and Bax mRNA/relative quantity of GAPDH mRNA [21].