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Docosahexaenoic acid confers enduring neuroprotection in experimental stroke☆

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ABSTRACT

Recently we demonstrated that docosahexaenoic acid (DHA) is highly neuroprotective when animals were allowed to survive during one week. This study was conducted to establish whether the neuroprotection induced by DHA persists with chronic survival. Sprague–Dawley rats underwent 2 h of middle cerebral artery occlusion (MCAo) and treated with DHA or saline at 3 h after MCAo. Animals received neurobehavioral examination (composite neuroscore, rota-rod, beam walking and Y maze tests) followed by *ex vivo* magnetic resonance imaging and histopathology at 3 weeks. DHA improved composite neurologic score beginning on day 1 by 20%, which persisted throughout weeks 1–3 by 24–41% compared to the saline-treated group. DHA prolonged the latency in rota-rod on weeks 2–3 by 162–178%, enhanced balance performance in the beam walking test on weeks 1 and 2 by 42–51%, and decreased the number of entries in the Y maze test by 51% and spontaneous alteration by 53% on week 2 compared to the saline-treated group. DHA treatment reduced tissue loss (computed from T2-weighted images) by 24% and total and cortical infarct volumes by 46% and 54% compared to the saline-treated group. These results show that DHA confers enduring ischemic neuroprotection.

1. Introduction

Stroke is a leading cause of death and disability in the United States. Annually, 795,000 people experience new or recurrent stroke and 41% of stroke patients die [1]. Even though some patients survive stroke, they suffer serious long-term disabilities such as a locomotion, sensory, vision, language and cognition. Thus, maximal enhancement of behavioral function accompanied with the reduction of infarction is a major goal of post-stroke therapy with a promise better quality of life for stroke survivors.

Docosahexaenoic acid (DHA; 22:6, $n = 3$) is a member of the essential omega-3 fatty acid family and is concentrated in the membranes of the central nervous system [2]. DHA is well known as a robust neuroprotectant against experimental stroke [3–5]. Recently we demonstrated that DHA improves behavioral function, decreases infarct volume, promotes cell survival in the ischemic penumbra as well as resolution of cerebral edema in one week of survival after focal cerebral ischemia in rats [3–5]. In addition, therapeutic window shows that DHA is neuroprotective when administered up to 5 h after experimental stroke during 7 days survival period [3]. The dose–response study in rats with transient focal cerebral ischemia showed that a 5 mg/kg dosage was highly neuroprotective [3–5]; thus, this dose was applied in this study.

The objective of the present study was to test the hypothesis that DHA-induced neuroprotection endures in animals allowed to survive for three weeks after focal ischemic insult. The effect of DHA treatment was investigated using a battery of different behavioral tests in conjunction with *ex vivo* magnetic resonance imaging (MRI) and histopathology.

2. Material and methods

2.1. Animal preparation

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Louisiana State University Health Sciences Center, New Orleans. Male Sprague–Dawley rats (290 to 330 g; Charles River Laboratory, Wilmington, MA, USA) were fasted overnight with free access to water before surgical procedure. Anesthesia was induced by inhalation of 3% of isoflurane in a mixture of 70% nitrous oxide and 30% oxygen. During the procedure, isoflurane was maintained at 1% in the same ratio of nitrous oxide and oxygen. Orally intubated rats were paralyzed by injection of pancuronium bromide (0.5 mg/kg, i.v.) and then mechanically ventilated during the surgical procedure. A catheter was inserted in the right femoral vein and then

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removed after infusion of the drug. **The femoral arterial catheter was not implanted and we did not measure arterial blood gases and glucose, to avoid functional impairment of the hindlimb**. Rectal (CMA/150 Temperature Controller, CMA/Microdialysis AB, Stockholm, Sweden) and cranial (left temporalis muscle; Omega Engineering, Stamford, CT, USA) temperatures were monitored and maintained at 37.0 to 37.5 °C during surgical procedures. Rectal temperature and body weight were closely monitored during the three-week survival period.

2.2. Focal cerebral ischemia model

The right middle cerebral artery (MCA) was occluded for 2 h by intraluminal filament, as we previously described [6]. Briefly, the right common carotid artery (CCA) was exposed through an incision in the neck. The CCA was isolated from surrounding nerves. The distal external carotid artery (ECA) and pterygopalatine arteries were tied. A 4-cm of 3–0 monofilament nylon suture coated with poly-Lysine was introduced via the proximal ECA into the internal carotid artery and MCA. After 2 h of MCAo, rats were anesthetized with the same anesthetic combination and intraluminal filaments were removed. The animals were allowed to survive for three weeks with free access to water and food.

2.3. Treatment

The agents (DHA; 5 mg/kg, Cayman, Ann Arbor, MI; $n = 12$) or vehicle (0.9% saline; $n = 8$) were administered intravenously into the femoral vein at 3 h after onset of MCAo.

2.4. Behavioral tests

Rats were pre-trained for the rota-rod, beam walking, and Y maze tests for three consecutive days before MCAo procedure. Animals that failed to accomplish the requirements of the tests during pre-training period were excluded from the study. All behavioral tests were performed by an investigator blinded to the treatment groups.

2.4.1. Total neurologic score

A composite neurological battery was conducted during MCAo (at 60 min) and then on days 1, 2, 3 and weeks 1, 2, and 3 after treatment. The battery consisted of two tests: (1) the postural reflex test to evaluate upper body posture when the animal is suspended by the tail, and (2) forelimb placing test to assess sensorimotor integration in forelimb placing responses to visual, tactile and proprioceptive stimuli [6]. Total neurologic score was graded on a scale from 0 (normal) to 12 (maximal deficit), as we previously described [6]. Only animals with a high-grade neurological deficit (10 or greater) were included in the study.

2.4.2. Rota-rod test

Rota-rod test was used to evaluate the motor function after cerebral ischemia [7]. In the pre-training period, animals were trained to run for 5 min at 16 RPM on a rota-rod (ENV-575, Med Associate, Inc., Albans, VT, USA). On weeks 1, 2 and 3 after treatment, the rota-rod test consisted of five trials per day on each test day. Each trial recorded the time to fall during a 5-min session at the speed of 16 RPM. A 15-min break was given between the five trials.

2.4.3. Beam walking test

The beam walking test was used to assess motor coordination, integration [8] and balance performance [9] after focal cerebral ischemia. Rats were pre-trained to cross the beam (100 cm × 2.5 cm × 2.5 cm; 60 cm above floor) voluntarily without a slip. The test was conducted three times per day on weeks 1, 2 and 3 after treatment.

Motor coordination and integration were assessed by counting the number of half and full slips on the ipsilateral and contralateral (stroke) sides, when the rat is traversing the beam for 3 min [10]. A step that slipped outside of beam was considered a full slip, while touching the side of the beam was defined as a half slip [11]. Balance performance on the beam was graded as follows: 0 = Balances with steady posture; 1 = Grasps side of beam; 2 = Hugs beam and 1 limb falls down from beam; 3 = Hugs beam and 2 limbs fall down from beam; 4 = Attempts to balance on beam but falls off > 40 sec; 5 = Attempts to balance on beam but falls off > 20 sec; 6 = Falls off, no attempt to balance or hang on beam < 20 sec [9]. Rats were given a 5-min break between trials.

2.4.4. Y maze test

The Y maze test was used to evaluate cognitive function, especially working memory in a new environment [12]. During the pre-training period, rats were allowed to explore the Y maze (Stoelting, Wood Dale, IL, USA) for 30 min. On weeks 1, 2 and 3 after treatment, the number of entries, spontaneous alteration and percentage of alteration were recorded for 8 min three times per test day. Entry is defined as a complete placement of hind paws within the arm of the maze. Spontaneous alteration is considered when a rat visits a new arm and does not return to one of previously-visited two arms. The percentage of alteration was calculated as follows: [Number of spontaneous alteration/(Number of entry − 2)] × 100 [12]. Rats were given a 10-min break between trials.

2.5. Ex vivo magnetic resonance imaging (MRI)

At three weeks after completion of the behavioral tests, brains were removed after transcardiac perfusion with 0.9% saline followed by 4% paraformaldehyde. High resolution *ex vivo* MRI data of brains were obtained using an 11.7 T Bruker Advance 8.9 cm horizontal bone instrument equipped with an 89 mm (ID) receiver coil (Bruker Biospin, Billerica, MA, USA). Lesion volume, tissue loss, left and right hemispheres areas and ventricles size were quantified from high resolution T2 weighted images (T2WI). Residual hemisphere volume on each side was calculated by subtraction of ventricle volume from the volume of the hemisphere on each side. Tissue loss was defined as the subtraction of residual volume of the lesion hemisphere from residual volume of the non-lesion hemisphere.

2.6. Histopathology

Histopathology was performed after completion of *ex vivo* MRI. Cryoprotected brains were cut into twenty-micron-thick sections in the coronal plane and stained with thionine (Nissl). Brain sections were then digitized (MCID core imaging software; InterFocus Imaging Ltd., Cambridge, England) at nine standardized coronal levels (bregma levels: +5.2, +2.7, +1.2, −0.3, −1.3, −1.8, −3.8, −5.0 and −7.3 mm) [13] using a CCD camera (QICAM Fast 1394, QIMAGING, British Columbia, Canada). An investigator blinded to the experimental groups then outlined the zone of the cortical and subcortical infarction as well as the left and right hemispheres of each section. Infarct volume was calculated as the integrated product of the cross-sectional area and intersectional distance. Brain sections were imaged on a motorized microscope BX61VS (Olympus, Japan) at 20 × objective.

2.7. Statistical analysis

Repeated measures analysis of variance (ANOVA), followed by Bonferroni procedure to correct multiple comparisons was performed. Two-tailed Student's *t*-tests were used for two-group comparisons. One-way ANOVA followed by Dunnett's test was used in the secondary analysis of the final behavioral endpoints. Data are presented as mean values ± SEM. Differences at $p < 0.05$ were considered statistically significant.