Treatment with docosahexaenoic acid after hypoxia-ischemia improves forepaw placing in a rat model of perinatal hypoxia-ischemia

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OBJECTIVE: Docosahexaenoic acid (DHA) is a dietary fatty acid with neuroprotective properties. We hypothesized that DHA treatment after hypoxia-ischemia would improve function and reduce brain volume loss in a perinatal rat model.

STUDY DESIGN: Seven-day-old Wistar rat pups from 7 litters (n = 84) underwent right carotid ligation, followed by 8% O_2 for 90 minutes. Fifteen minutes after hypoxia-ischemia, pups were divided into 3 treatment groups (intraperitoneal injections of DHA 1, 2.5, or 5 mg/kg) and 2 control groups (25% albumin or saline). At 14 days, rats underwent vibrissae-stimulated forepaw placing testing, and bilateral regional volumes were calculated for cortex, striatum, hippocampus, and hemisphere.

RESULTS: Posthypoxia-ischemia treatment with DHA acid significantly improved vibrissae forepaw placing (complete responses: 8.5 ± 2 treatment vs 7.4 ± 2 controls; normal = 10; $P = .032$, t test). Postinjury DHA treatment did not attenuate brain volume loss in any region.

CONCLUSION: Posthypoxia-ischemia DHA treatment significantly improves functional outcome.

Key words: docosahexaenoic acid, hypoxia-ischemia, neuroprotection, perinatal rescue therapy

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In a recent systematic review, 14% of cases of cerebral palsy among nonanomalous term infants were associated with intrapartum hypoxia-ischemia (HI).[1] Neonatal hypoxic-ischemic encephalopathy (HIE) complicates approximately 2-9 per 1000 live births.[1-3] Although the majority of infants with mild HIE will develop normally, approximately one-third to one-half of infants with moderate HIE and the vast majority of surviving infants with severe HIE will ultimately be found to have neurodevelopmental disability.[4,5] Many recent investigations have focused on the preclinical development of strategies to rescue the immature brain after a HI insult. Perinatal HI brain injury involves an acute injury phase, followed by a reperfusion phase with a 6- to 8-hour therapeutic window during which there is potential for attenuation of perinatal brain injury.[6,7] Investigators have studied a number of agents with post-HI neuroprotective properties, including allopurinol, magnesium sulfate, simvastatin, erythropoietin, isofluorane, topiramate, and matrix metalloproteinase inhibition.[6,8] Most of these neuroprotective modalities target a particular stage of the brain injury cascade, but therapeutic use of several of these agents is limited by safety or toxicity considerations.[6,8] Of measures designed for posthypoxia "rescue" treatment, only therapeutic hypothermia has been tested in large randomized controlled trials and has entered into neonatal clinical practice[9,10] (NNT = 7).

Because of its well-established safety record and its ability to readily cross the blood-brain barrier, docosahexaenoic acid (DHA) is an especially promising neuroprotective agent.[11] DHA is a long-chain polyunsaturated fatty acid available in the diet. Along with its major metabolite, Neuroprotectin D1, DHA has been shown to have neuroprotective properties in animal models of adult brain and spinal cord injury.[12-16] DHA may simultaneously attenuate several stages of the brain injury cascade, including bioactive lipid mediators, free radicals, inflammatory cytokines, and apoptosis.[11,12,17] We have previously demonstrated that DHA pretreatment improves function and reduces brain volume loss in a rodent model of perinatal HI.[18] We have chosen to use the Rice Vannucci model, a highly characterized model of perinatal hypoxemia that is well suited for assessment of neurodevelopmental outcomes and neuropathology.[19] For our studies to date, we have chosen a regimen that incorporates DHA complexed to albumin as originally described by Belayev et al in an adult stroke model.[11] The objective of the current investigation is to test whether "rescue" treatment with DHA after HI will improve neurologic function and reduce volume loss in a rodent model simulating perinatal HI.

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Materials and Methods

Preparation of DHA-albumin complex

DHA (D2534 as cis-4,7,10,13,16,19-DHA; Sigma-Aldrich, St. Louis, MO) was complexed to human albumin by incubating 4 mL human serum albumin 25% (Baxter, Deerfield, IL) with 4 mg DHA to yield a final concentration of DHA 25 mg/25 μL. Each vial was aliquoted in 1-mg/mL samples and kept under nitrogen in a −20°C freezer. Nitrogen was reapplied to the vials weekly.

Animals

Postnatal day 7 (P7) Wistar rats were obtained in litters adjusted to equal sex distribution (Charles River Laboratories, Portage, MI). Animals were treated in accordance with protocols approved by our University Committee on the Use and Care of Animals in research. Pups were housed with the dam and littermates throughout the duration of the experiments.

To test the effect of post-HI DHA treatment, 7-day-old (P7) Wistar rat pups from 7 litters (n = 84) were randomly divided into 3 treatment groups and 2 control groups. Treatment groups received intraperitoneal (IP) injections of DHA 1 mg/kg, 2.5 mg/kg, or 5 mg/kg as DHA-albumin complex. Control groups received a similar volume of 25% albumin or normal saline (NaCl). There were at least 2 pups (1 male and 1 female) in each litter from each of the 3 treatment groups and each of the 2 control groups. Because each litter contained 12 animals, each litter included 4 animals from 1 of the 5 groups, which varied from litter to litter to balance out the sample size among treatment groups across all litters.

P7 pups anesthetized with isoflourane (induction at 3.5%, maintenance at 1.5%) underwent right common carotid artery double ligation and division through a ventral neck incision. Pups were returned to their dams and recovered for 1.5 hours at 37°C. After carotid ligation and recovery, HI was induced by placing the pups in 500 mL jars partially submerged in a water bath at 36°C in 8% oxygen for 90 minutes to induce unilateral cerebral HI. In this model, unilateral carotid ligation alone or 90 minute hypoxia exposure alone do not produce detectible brain injury.[20] However, unilateral carotid ligation, followed by timed hypoxia exposure induces ipsilateral cerebral ischemia during the hypoxia exposure, followed by reperfusion immediately after return to room air.[21] Fifteen minutes after HI, the pups received control or treatment IP injections. Once normal activity was resumed, the pups were returned to the dam where they remained until P14. Pups were weighed before HI on P7 and subsequently on P14.

Vibrissae stimulated forepaw placing test

On P14, we evaluated sensorimotor outcome using the vibrissae-stimulated forepaw placing test, as previously described.[18] This test is a quantifiable functional measure of injury to the contralateral sensorimotor cortex or striatum.[22] Briefly, stimulation of the vibrissae (whiskers) on a surface edge results in extension of the forepaw on the same side as the stimulated whiskers to reach the stimulating surface, in a complete response. At P14, the typical response contralateral to the nonlesioned hemisphere is immediate extension of the forepaw to contact the stimulating surface in 10 of 10 trials. In a partial response, the forepaw is incompletely extended without contacting the stimulating surface. A weighted vibrissae score to incorporate data from both complete and partial responses was calculated using the formula [partial contacts + 2 * (complete contacts)].

Histopathology

Severity of brain injury was evaluated on P14 by calculating volume of tissue with intact staining using ImageJ software (US National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/), in regularly spaced cresyl violet stained coronal sections, as previously described.[18] Volumes were calculated from hemispheric and regional (cortex, striatum, hippocampus, and "other") area measurements in regularly spaced frozen coronal 20 μm sections. Volume was estimated by summing areas and multiplying by the distance between sections. The "other" region incorporated all remaining intact tissue in the hemisphere other than cortex, striatum, or hippocampus and included thalamus, septum, fimbria-fornix, corpus callosum, and other major white matter tracts. Thus, hemisphere area in each section was the sum of cortex, striatum, hippocampus, and "other".

Statistical analysis

A linear mixed models analysis of variance was used to evaluate differences in percent damage for hemisphere and each brain region among groups. We used litter as a random effect, treatment, and sex as fixed effects, and evaluated treatment by sex interaction. A similar linear mixed model was used to compare forepaw placing successes among treatments. Post hoc comparisons of treatment group means were carried out using the Fisher protected least significant difference test. The simplest model containing only treatment as a predictor with litter as a random effect was obtained. Our sample size (16-17/group) was chosen both based on our prior experience with DHA treatment before HI and on a sample size calculation in which 16 subjects/group is sufficient to detect a between-group difference of 1 standard deviation with a power of 0.8 and alpha of .05.

Results

Survival/morbidity

Of the 84 pups that underwent the HI procedures, 77 survived (91%) during the experiments. In 1 litter, after 7 days of housing, the dam appeared unwell. Within this litter, 5 of the 12 pups had died by P14; the remainder appeared unwell and unusually small. Because of concerns regarding dam and litter health, statistical analysis did not include this litter. Two pups from another litter died between P7 and P14, and were excluded from analysis of pathology and function (1 received albumin, the other received NS). Final data analysis included 70 animals.

Body weights

There were no differences in initial weight or weight change from P7 to P14