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## Docosahexaenoic acid Confers Neuroprotection in a Rat Model of Perinatal Hypoxia-ischemia potentiated by *E. coli* lipopolysaccharide-induced systemic inflammation

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### Abstract

**Objective**—Lipopolysaccharide (LPS) pretreatment potentiates HI injury. We hypothesized that docosahexaenoic acid (DHA) pretreatment would improve function and reduce brain damage in this rat model of perinatal brain injury and inflammation.

**Study Design**—Seven-day-old Wistar rats were divided into 3 groups. One received intraperitoneal (IP) DHA 1 mg/kg and LPS 0.1mg/kg. The second received 25% Albumin and LPS. The third received normal saline (NS). Injections were given 2.5 hours prior to right carotid ligation, followed by 90 minutes 8% O<sub>2</sub>. Rats underwent sensorimotor testing and brain damage assessment on P14.

**Results**—DHA pretreatment improved forepaw placing compared to albumin/LPS. (Mean±SD successes/10 trials: 8.57±1.7 DHA/LPS vs 6.72±2.2 Albumin/LPS,  $p<.0009$ ). There were no significant differences in brain damage among groups.

**Conclusions**—Inflammatory stimulation before HI resulted in poorer function than HI alone. Although DHA pretreatment had no impact on brain damage, it significantly improved function in neonatal rats exposed to LPS and HI.

### Keywords

Docosahexaenoic acid; hypoxia ischemia; neonatal; neuroprotection; inflammation

### Introduction

Intrapartum hypoxia-ischemia (HI) may lead to neonatal encephalopathy and to permanent neurologic disability.[1] Electronic fetal monitoring (EFM) was introduced in the 1970s

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with the expectation that recognition of intrapartum hypoxia and timely intervention would reduce cerebral palsy by as much as 50%.[2] Unfortunately, use of EFM failed to reduce cerebral palsy in large randomized controlled trials,[3] and the widespread use of EFM and expedited delivery in developed countries has not reduced the incidence of cerebral palsy among term infants. [1] One possible reason for the ineffectiveness of EFM in preventing cerebral palsy may be the complex and previously poorly understood etiology of neurologic injury.

Recently, the role of inflammation and the fetal systemic inflammatory response syndrome in the etiology of cerebral palsy has been recognized.[4-6] Using a large California cord blood repository, Nelson demonstrated that increased levels of interleukins (ILs) 1,6,8,9,11,13 and tumor necrosis factor- $\alpha$ , were present in cord blood taken from term infants destined to develop cerebral palsy compared to healthy controls. [4] Similarly, in a prospective cohort study of 123 preterm infants born to mothers who underwent amniocentesis, Yoon demonstrated that elevations of amniotic fluid IL 6 and 8 as well as histologic funisitis were strongly associated with the diagnosis of cerebral palsy at 3 years of age.[5] A population based study demonstrated that the coexistence of a potentially asphyxiating condition, such as tight nuchal cord and maternal infection, conferred a much higher risk of spastic quadriplegic cerebral palsy together than either condition alone. [7] Drawing on these observations, Peebles hypothesized that inflammation lowers the threshold at which intrapartum hypoxia results in neurologic injury. [8] This improved understanding of the interaction between hypoxia and inflammation suggests new approaches to perinatal neuroprotection.

One such novel approach is docosahexaenoic acid, (DHA), a long chain polyunsaturated fatty acid. DHA is an integral component of neuronal cell membranes and synaptic terminals.[9] DHA is readily available in the diet in fish and algae, and epidemiologic observation suggests that maternal diets rich in fish are associated with reduced risk for cerebral palsy.[10] In adult rodent models of brain ischemia-reperfusion and spinal cord injury, DHA has been shown to exert neuroprotective effects and to improve functional outcome.[11,12] DHA may exert anti-inflammatory effects by altering the presentation of Toll-like receptor 4, the lipopolysaccharide (LPS) receptor, on the microglial cell membrane, thereby modulating the cyclooxygenase-2 signaling pathway, and reducing proinflammatory cytokine production.[13-15] DHA is the metabolic precursor of D-series resolvins and neuroprotectins. Neuroprotectin D1 attenuates NF  $\kappa$ B production and COX2 expression, reduces influx of polymorphonucleocytes, and counters apoptosis, thus promoting neural cell survival.[9,16] D-series resolvins block TNF- $\alpha$  induced IL-1 $\beta$  transcripts in microglial cells and limit PMN infiltration into inflamed brains. [9]

In our earlier experiments using a neonatal (P7) rat model of perinatal hypoxia-ischemia, we have shown that DHA pretreatment with 1 mg/kg, 2.5 mg/kg, and 5 mg/kg doses reduces brain volume loss and improves neurologic functioning as measured by the vibrissae stimulated forepaw placing test.[17] In those dose-finding experiments, we demonstrated that the 1 mg/kg dose was most neuroprotective.[17]

The objective of this second series of experiments is to test DHA pretreatment in a neonatal rat (P7) model of hypoxia-ischemia potentiated by inflammation. We hypothesized that DHA pretreatment would reduce brain volume loss and improve neurologic functioning in an animal model of perinatal HI with inflammation that may more nearly reflect the conditions leading to cerebral palsy than HI alone.

## Materials and Methods

### Preparation of DHA-Albumin Complex

DHA was delivered as a powder (Sigma, St. Louis, Mo, Cat#: D2534 as cis-4,7,10,13,16,19-DHA. DHA was complexed to human albumin by incubating 4mL of human serum albumin 25% (Baxter, Deerfield, IL) with 4mg of DHA to yield a final concentration of DHA 25mg/25 $\mu$ L. Each vial was aliquoted in 1-mg/mL samples and kept under nitrogen in a  $-20^{\circ}\text{C}$  freezer. Nitrogen was reapplied to the vials weekly.

### Animals

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 DHA pretreatment, we used a modification of the LPS pretreatment model described by Eklind.[18] Rats received intraperitoneal *Escherichia coli* lipopolysaccharide (LPS) 055:B5 (0.1 mg/kg) (Sigma, St. Louis) followed 2.5 hours later by right carotid artery ligation. Ninety minutes after ligation, the animals were then placed in the hypoxia chambers partially submerged in a water bath and then returned to the dams at  $36^{\circ}\text{C}$  for 90 minutes. In contrast to the Eklind model, we chose the 90 minute duration of hypoxia for consistency with our prior studies with DHA using HI alone. Based on our prior dose finding studies[17], we used the 1 mg/kg dose of DHA for pretreatment in the current experiments. Sample size calculations indicate that 16–17 subjects/group are needed to detect a reduction in percent damage of one standard deviation, with  $\alpha=0.05$ ,  $\beta=0.8$ .

Based on these calculations, six litters (N=72) of Wistar pups all underwent both hypoxic ischemia and treatment interventions on P7. Within each litter, pups were divided into three groups. The first group received intraperitoneal (IP) DHA 1mg/kg as DHA-albumin complex immediately followed by IP LPS (0.1mg/kg) [DHA/LPS]. The second group received IP 25% albumin immediately followed by IP LPS [ALB/LPS]. The third group received IP normal saline (NS) immediately followed by IP NS (0.1mg/kg) [NS/NS]. This third group [NS/NS] was used as a control to provide lesioning conditions within the experiments from which we could compare histopathologic lesions in a group of pups undergoing HI alone without an inflammatory stimulus (LPS).

Pups received the initial IP injection (5 $\mu$ L/g body weight) of DHA, 25% albumin or NS followed immediately by their LPS or NS injections. Pups were returned to their dams and allowed to recover. At 2.5 hours after injection, pups were anesthetized with isoflourane (induction at 3.5%, maintenance at 1.5%) and the right common carotid artery was divided after double ligation with surgical silk through a ventral neck incision. Pups then recovered for 1.5 hours (30 minutes in a  $37^{\circ}\text{C}$  incubator then 60 minutes with the dam). After carotid ligation and recovery, HI was induced by placing the pups in 500mL glass jars partially submerged in a water bath at  $36^{\circ}\text{C}$  in 8% oxygen for 90 minutes to induce unilateral cerebral HI. After HI, pups recovered in a  $37^{\circ}\text{C}$  incubator for 15–30 minutes. Once normal activity was resumed, the pups were returned to the dam where they remained until P14.

Animal rectal temperatures were obtained using a 0.6-mm flexible temperature probe (YSI-400 Tele-Thermometer, Yellow Springs Instruments, Yellow Springs, OH) before injection, after injection, before and after carotid ligation, before and after HI, 1 hour after HI and on P14. Pups were weighed before HI on P7 and subsequently on P14. The temperatures and weights were obtained both as representative measures of morbidity and to

document that there were no temperature effects of the treatments, i.e. hypothermia that could affect or improve outcomes.

### **Vibrissae Stimulated Forepaw Placing Test**

We chose the Vibrissae-stimulated forepaw placing test as a readily quantifiable functional measure of injury to the sensorimotor cortex or striatum.[19] The vibrissae test was performed on P14 pups in the following manner. Stimulation of the vibrissae (whiskers) on a surface edge results in extension of the forepaw on the same side as the simulated whiskers to reach the stimulating surface. In a partial response, the forepaw is incompletely extended without contacting the stimulating surface. Therefore, stimulation of the left vibrissae can demonstrate left forepaw placement deficits as a consequent of right sided hypoxic ischemic injury. As the right hemisphere controls left sided motor function, stimulation of the left vibrissae tests the right hemisphere. Stimulation of the right vibrissae, contralateral to the intact hemisphere, tests the unaffected cerebral hemisphere. A weighted vibrissae score to incorporate data from both complete and partial responses was calculated using the formula [partial contacts + 2 \* (complete contacts)].

### **Histopathology**

P14 rats were deeply anesthetized and euthanized followed by brain removal. Frozen coronal 20 $\mu$ m sections were stained with cresyl violet. Severity of brain injury was evaluated by calculating volume of tissue with intact staining using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>). Volumes were calculated from hemispheric and regional (cortex, striatum, hippocampus, and “other”) area measurements in regularly spaced sections. The “other” region included thalamus, septum, fimbria-fornix, corpus callosum, and major white matter tracts. Damage severity (percent tissue loss) of right sided injury was calculated as previously described. [17]

### **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 evaluate forepaw placing successes among treatments. Post hoc comparisons of treatment group means were carried out using the Tukey-Kramer adjustment for multiple comparisons. The effect of LPS on mortality was evaluated by analysis of variance with treatment as a fixed effect and litter as a random effect. NS/NS control pups were excluded from subsequent mortality analysis as there were no deaths in this group. To evaluate an effect of DHA on mortality among all LPS treated pups, we used a generalized mixed model with treatment as a fixed effect and litter as a random effect in order to assess the probability of dying in the ALB/LPS and DHA/LPS treatment groups only.

## **Results**

### **Survival/Mortality**

Of the seventy two rats that underwent the HI procedures, 16 pups (22%) died during the experiments. Of these, 11 died during HI, 2 died by injury by the dam immediately after HI and 3 died in the home cage between days P7 and P14. All of the 16 pups who died had received LPS. LPS treated rats were more likely to die than those who received NS. ( $p=.0013$ ). Among the LPS treated rats, there was no difference in mortality between those

treated with DHA compared to those treated with albumin (DHA/LPS 10/24 vs ALB/LPS 6/24;  $p=.17$ ,  $f_{1,40}$ ).

### Body Weights and Temperatures

There were no significant differences between DHA/LPS, ALB/LPS and NS/NS groups with respect to rectal temperatures at any time-point. There was not a significant difference between the groups with respect to pretreatment body weight. Similarly, there were no significant differences in P7 or P14 body weights or in weight change from P7 to P14 among the three treatment groups. In addition, there was no difference in weights between the combined LPS groups and the NS pretreated pups.

### Vibrissae Stimulated Forepaw Placing Test

Seven days after injection and HI, the surviving 56 animals underwent the lateral vibrissae-stimulated forepaw placing test. The vibrissae scores of the ALB/LPS rats were significantly worse than those of the NS/NS rats (See Fig A:  $13.83 \pm 0.82$  versus  $17.21 \pm 0.071$ ;  $p=.0076$ ), demonstrating that LPS treatment potentiated the functional deficit induced by HI. Among LPS pretreated rats, DHA/LPS rats had significantly better left forepaw placement results when compared to the ALB/LPS group. (See Fig A:  $17.72 \pm 0.92$  versus  $13.83 \pm 0.82$ ;  $p=.007$ , Tukey adjustment). Furthermore, forepaw placing scores of the DHA/LPS pups were not different than scores of the pups that underwent HI but did not receive LPS (NS/NS). (See Fig A;  $p=.893$ , Tukey adjustment). There was a borderline effect of sex on vibrissae response with females performing better than males ( $17.16$  vs.  $15.36$ ,  $p=.059$ ), but there was no treatment by sex interaction. No litter effects on forepaw placing responses were seen. Right vibrissae stimulation consistently elicited correct right paw placement 10 out of 10 trials in all groups. Results of the vibrissae forepaw placing tests are summarized in Figure 1.

### Histopathology

Ninety minutes of HI resulted in moderately severe lesions as demonstrated by the NS/NS group who had striatal and hippocampal atrophy, cortical thinning and/or cystic infarction and an overall 26% reduction in right hemisphere volume compared to intact left. No statistically significant differences are seen when comparing the hemisphere or regional volumes or percent damage between the DHA/LPS group and the ALB/LPS group. Additionally, no statistically significant differences were seen when comparing these same measures in the LPS treated groups (DHA/LPS and ALB/LPS) vs the group that did not receive LPS (NS/NS). Histopathology results are summarized in Table 1.

### Comment

The objective of our study was to examine whether pretreatment with DHA blocks potentiation of HI injury by Lipopolysaccharide (LPS) induced systemic inflammation in neonatal rats. Our findings are similar to those described by Eklind in that the addition of an inflammatory stimulus (LPS) to the perinatal HI model places neonatal rat pups at significant increased mortality risk.[18] In our study, DHA did not improve the pups' rates of survival. However, among survivors, function was improved (i.e. impairment was significantly decreased) by DHA-Albumin pretreatment compared to the pups who received albumin alone ( $p=.007$ ). In fact, DHA treatment before HI/LPS improved the vibrissae-stimulated forepaw placing response to the level observed in neonatal rats exposed to HI alone (NS/NS). That could be interpreted to mean that DHA treatment reversed the potentiation of HI injury by LPS.

Our study used intraperitoneal injection of E. Coli Lipopolysaccharide as a model for intrapartum chorioamnionitis preceding HI conditions producing moderate brain lesion severity. In contrast to the original description of the model by Eklind,[18] the observed percent brain volume loss in rats exposed to HI and LPS in our experiments was not statistically different than that observed in rats exposed to HI alone. This lack of potentiation of damage severity may have resulted from the modification we made to the Eklind model, increasing hypoxia duration to 90 minutes for all animals. Eklind, et al demonstrated that LPS pretreatment changes the injury threshold with short hypoxia durations without significantly changing injury severity at longer hypoxia durations. Our histopathologic findings, with relatively longer hypoxia duration, are consistent with the latter observation. Nonetheless, vibrissae scores for rats receiving LPS were significantly worse than those exposed to HI alone, suggesting potentiation of hypoxic ischemic injury by inflammation.

There are several possible explanations for this disconnect between the effect of LPS on functional outcome and its effect on histopathology in our experiments. In a rat model exploring prenatal risk factors for later development of Parkinson's Disease, prenatal exposure to LPS alone was demonstrated to significantly reduce dopamine and serotonin in the frontal cortex, striatum, amygdala, hippocampus, nucleus accumbens, and striatum.[20] LPS alone is toxic to dopaminergic neurons in the nigrostriatal pathways involved in regulation of motor function. [20-22] In another rat model, prenatal LPS exposure resulted in alterations in microglial morphology signifying a more advanced stage of microglial activation compared to normal saline exposure.[21] Ling et al demonstrated effects of microglial activation on motor function that persisted over the lifespan of the rats. These potential mechanisms may explain the significant worsening of vibrissae forepaw placing scores in LPS-exposed animals in our model in the absence of brain volume difference. This is consistent with reported limited utility of brain volume measurements in various regions in predicting clinical outcome in human infants. [23]

Similarly, in our model of HI potentiated by LPS, DHA-Albumin pretreatment significantly improved function as measured by the vibrissae-stimulated forepaw placing test compared to albumin alone, although no reduction in brain volume loss was seen. Potential mechanisms whereby DHA might improve functional outcome independent of observed brain volume include enhancement of synaptic signal transduction, incorporation into neural cell membranes thereby increasing membrane fluidity, and modulation of dopaminergic and serotonergic neurotransmitter function.[24-26] DHA may also have preserved the integrity of essential neural tracts without affecting the overall brain volume loss. In a rodent model of spinal cord injury, DHA was shown to reduce microglial activation and macrophage recruitment, and to preserve myelination.[27]

A limitation of these preliminary experiments is that we have not explored cellular and molecular mechanisms whereby DHA improves functional outcome. Similarly, we have not yet assessed the long-term durability of these beneficial effects. More studies of long-term outcomes after DHA pretreatment are needed before DHA will be ready to be translated into human clinical trials. For the future, an additional important question is whether similar improvements would be observed after maternal dietary DHA supplementation.

Combining the results of the present experiments and our previous studies, we have demonstrated that DHA pretreatment improves functional outcomes after HI alone[17] and after HI potentiated by LPS. There is clearly a discordance between functional and structural evidence of benefit. However, in terms of translation to human studies, the outcome of most clinical interest and significance would be function, not neuropathology.

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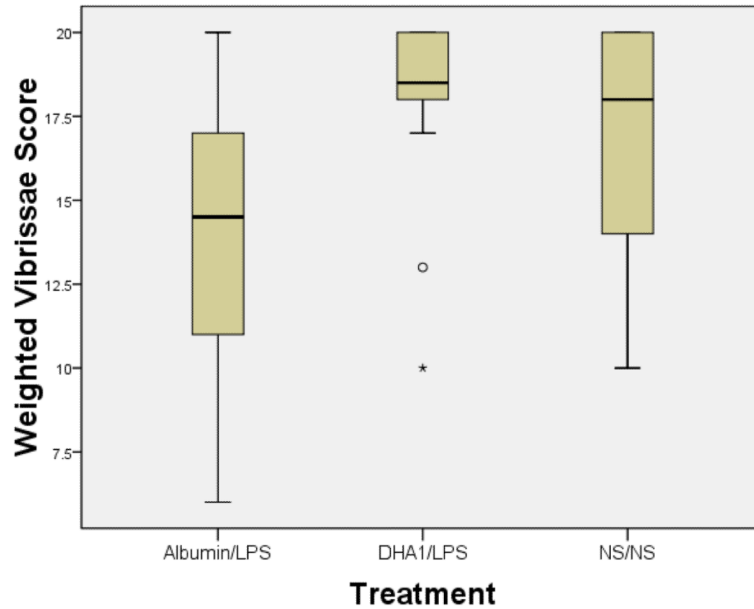
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**Figure 1. DHA pretreatment attenuates forepaw placing deficits among hypoxic ischemic pups exposed to LPS pretreatment**

Vibrissae-stimulated forepaw placing test results in control groups (that received normal saline followed by normal saline) and treatment groups (that received DHA 1mg/kg or Albumin followed by *E. coli* lipopolysaccharide inflammation) prior to undergoing right (R) carotid ligation followed by 90 minutes of hypoxia at 8% oxygen on postnatal day (P) 7. Testing was performed on P14 (10 trials/side). The y-axis = weighted response score in which complete forepaw placing on stimulus surface received score of 2 and partial response (i.e. forepaw motion without placing on stimulus surface) received score of 1. Best possible weighted score is 20. Boxes= interquartile range; horizontal bars=medians; whiskers=SE. They extend to data points no more than 1.5 width of box. All animals consistently responded to R vibrissae stimulation with appropriate R paw placement. Impairment of left (L) paw placement in response to L vibrissae stimulation was seen in Albumin/LPS rats compared to normal saline/normal saline rats. Among LPS pretreated groups, performance was significantly better in the DHA/LPS treatment group when compared to Albumin/LPS ( $P=.007$ ; Tukey-Kramer test).

**Table 1**

Effect of Docosahexaenoic acid pretreatment on severity of brain volume loss by region

Treat	N	Hippocampus	Other*	Cortex	Striatum
DHA/LPS	14	33.8±23.1	14.3±8.5	20.4±13.1	25.6±10.7
ALB/LPS	24	31.6±29.7	14.3±12.8	24.8±24.3	23.3±15.9
NS/NS	18	38.8±38.2	20.9±18.9	31.3±27.9	29.1±21.3

DHA, docosahexaenoic acid; LPS, Escherichia coli lipopolysaccharide; NS, normal saline. Values are means±standard deviation of regional percent damage, calculated from regional volumes using the formula  $100 * (L-R) / L$

\* other refers to any forebrain tissue other than hippocampus, cortex or striatum