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Abstract

Introduction: Up to 84% of prematurely born infants suffer hypoxic, anoxic, and ischemic insults. Those infants with subsequent behavioral, motor or cognitive dysfunction represent 8–11% of all live births. Yet, no interventions employed during pregnancy attenuate risk of morbidity in those at-risk infants. Dietary supplementation with omega-3 polyunsaturated fatty acids (ω-3 PUFAs) has been shown to reduce stroke-induced neuropathology in rat models emulating this adverse clinical event. To extend those studies we sought to determine whether maternal dietary supplementation with ω-3 PUFAs would confer neuroprotection against hypoxia-induced neurochemical dysfunction in newborn rat pups exposed to repetitive hypoxic insults.

Methods: We provided pregnant rats with either a ω-3 PUFAs enriched diet or else a standard rat chow diet. At postnatal day 7, pups were sacrificed and brain dopamine levels characterized. Results: Baseline brain dopamine levels did not differ between rat pups born to dams who received ω-3 PUFAs enriched versus standard rat chow diets. Rat pups born to dams maintained on normal diets, who were exposed to five days of repetitive hypoxic insults, experienced a 57% reduction in striatal dopamine levels accompanied by significant apoptosis. In contrast, ω-3 PUFAs-enriched newborn pups experienced no loss in striatal dopamine levels, and only minimal apoptosis. Conclusions: Our findings suggest that it may be feasible to confer neuroprotection against hypoxia-induced dopamine dysfunction to newborns likely to experience hypoxic insults. This could significantly improve the outcomes of those 8–11% of newborns who would otherwise experience hypoxia-induced behavioral, motor and cognitive dysfunction.

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1. Introduction

Hypoxic insults occurring during the perinatal period are among the leading causes of permanent brain dysfunction and remain a serious public health concern [1–3]. Most hypoxic insults typically occur in the setting of unambiguous clinical compromise, such as placental dysfunction, prolonged labor, or cardiopulmonary resuscitation [4,5]. However, other more insidious mechanisms, such as apnea of prematurity, which is a common occurrence in prematurely born infants [6–9], can induce repetitive hypoxic insults. Between 10% and 13% of all infants are born prematurely [10,11]. Apnea with concomitant hypoxic insults will afflict 78% of those born at 26–27 weeks gestation, 54% born at 30–31 weeks, and 7% born at 34–35 weeks [6].

Regardless of whether children who are born prematurely or at term, perinatal hypoxic insults are associated with diminished academic performance and other manifestations of executive dysfunction [12–16]. Perturbed function within neural networks subserving arousal and/or vigilance is also seen; infants with a history of apnea of prematurity require more intensive stimuli to be awakened from sleep [17]. Dampened autonomic dysfunction, manifesting as higher resting heart rates with reduced heart rate...
variability, has also been reported [18]. Yet despite these adverse outcomes attributed to perinatally-occurring hypoxic insults, there has been little progress towards developing clinical interventions that can be initiated during pregnancy to confer neural resiliency to at-risk newborns [19]. The absence of such interventions underlines progress towards mitigating the morbidity and mortality associated with these adverse perinatal events.

An emerging body of literature suggests that omega-3 polyunsaturated fatty acids (ω-3 PUFAs) confer neural resiliency against a number of insults [20–23]. Yet, there remains a paucity of data describing whether dietary supplementation with ω-3 PUFAs can confer neuroprotection against hypoxic insults occurring in their newborns. To overcome this barrier, this study was designed to characterize the extent that maternal prenatal dietary supplementation with ω-3 PUFAs will confer neuroprotective resiliency to the newborn against hypoxia-induced dopamine dysfunction, one neurotransmitter system that is exquisitely vulnerable to such insults [24,25].

2. Material and methods

All studies were performed at Emory University under an IACUC approved protocol (170–2003). Pregnant rats received dietary supplementation with ω-3 PUFAs by adding menhaden fish oil 15% weight by weight (w/w) (Sigma-Aldrich) into standard rat Chow mix, to achieve a total daily dose of 3.5–4.0 g. The ω-3 PUFAs enriched diet was initiated on day 1 of pregnancy (confirmed by presence of vaginal plug) and continued through 12 days post-delivery of their pups. Therefore, ω-3 PUFA enriched pups received both pre- and postnatal (via maternal colostrum) dietary enrichment with ω-3 PUFAs. Control rats were born to dams that were maintained on a standard rat Chow diet during pregnancy and thereafter.

Beginning on postnatal (PN) day 7 and continuing through PN 12, both ω-3 PUFA-enriched and control newborn pups were assigned randomly to receive either repetitive hypoxic insults or bursts of compressed room air, as described below, for 2 h blocks of time. At the conclusion of each 2 h period, all pups were returned to the dam for a 45 min interval, for feeding and grooming. This sequence was repeated 2 more times each day, totaling three 2-hour sessions of repetitive hypoxic insults or bursts of compressed air. During PN 7–12, pups were exposed to either a total daily dose of 6 h of repetitive bursts of hypoxia or normoxia. On PN 12, at the conclusion of the protocol, pups were euthanized for neurochemistry. Our protocol for inducing repetitive hypoxic insults has been previously validated (23) to insure that it does not induce maternal separation-induced stress in newborn rat pups, which can evoke changes in neurochemistry [26–30].

2.1. Hypoxia-inducing system

Our hypoxia-inducing system consists of a clear Plexiglas chamber, solenoid valves, and compressed gas [24,25]. The internal environment within the system is warmed and humidified to the appropriate level for each postnatal day of age [31,32]. Attached to the hypoxia-inducing chamber is one gas cylinder containing 10% oxygen, 3% carbon dioxide, and nitrogen and a second gas cylinder containing only compressed air. After placing the rats into the chamber, a programmable timer opens the solenoid valve between the hypoxic gas cylinder and hypoxic chamber for a 20 s period, allowing introduction of the hypoxic gas mixture at a flow rate of 10 liters per minute, which provides 18.5 complete air exchanges per minute within the chamber. As the solenoid valve closes, the second solenoid valve, attached to the gas cylinder containing only compressed air, opens for 40 s. These alternate on and off to expose the rats to 20 s bursts of hypoxia, followed by 40 s bursts of room air, thereby inducing 60 hypoxic events per hour. This novel system allows for a user-selected frequency and duration of hypoxic insults, thereby permitting us to determine the specific hypoxic “doses” delivered. In addition to the hypoxia-inducing chambers, we also use identically constructed normoxic chambers. Solenoid valves of these chambers are attached to cylinders containing only compressed air, which cycled through the chambers at the same flow rates and frequency as gas flowed through the hypoxia-inducing chamber. The normoxia chamber acted as a control for rats receiving hypoxic gas and for any potential impact associated with maternal separation [26–29]. Additionally, the duplicate use of solenoid valves and a compressed gas source on the normoxia chamber allow further control of other factors such as sound, pressure changes, or temperature fluctuations, all of which are intrinsic to compressed gas sources.

2.2. HPLC assessment of brain tissue content of dopamine

Briefly, rats were decapitated and neural tissue was dissected from the precommissural striatum at coordinates previously described [24]. Dissected neural tissue was homogenized, centrifuged at 10,000 rpm for 10 min, and then filtered through a 0.22 μm filter. Samples were then placed in our refrigerated autosampler which injected them into our high performance liquid chromatography (HPLC) system. Chromatography. Homogenates were assayed for dopamine using HPLC with electrochemical detection as previously described [25]. Twenty microliter samples were injected from the autosampler onto a C18 reversed phase column maintained at 30 °C. De-gassed mobile phase was delivered at a flow rate of 0.3 mL/min. The electrochemical detection was performed by a GBC Antec Leyden VT03 electrochemical flow cell with a glassy carbon working electrode maintained at a potential of +0.60 V, relative to the reference electrode.

2.3. Immunohistochemistry for apoptosis

Rat pups were deeply anesthetized with a lethal dose of sodium pentobarbital, perfused transcardially with 0.9% heparinized-saline followed by a fixative of 4% paraformaldehyde. Brains are removed, equilibrated overnight in 30% sucrose, and sectioned on a freezing microtome at a thickness of 50 μm. Sections were collected in 0.05 M Tris-buffered saline containing 1% sodium azide. Adjacent series are processed for TH or Nissl substance using neutral red or thionin. For caspase-3 processing, sections were incubated 24–48 h at 4 °C with primary antibody polyclonal rabbit-anti-caspase 3 in diluent of normal goat serum, triton X-100 and TBS. Following incubation and three 5 min rinses in TBS, sections were incubated for 1.5–2 h in secondary biotinylated goat-anti-rabbit, rinsed in TBS and incubated in avidin-biotin-peroxidase complex in TBS and Triton X-100. Sections were then incubated in 0.05% 3,3 diaminobenzidine tetrachloride (DAB) and 0.01% hydrogen peroxide in 0.05 M Tris buffer for 5–10 min. The reaction was stopped by extensive rinses in TBS. Brain sections were mounted onto gelatin-coated slides, air dried, dehydrated in ethanol, cleared in xylene and coverslipped with DPX mountant using procedures employed within our laboratory [33].

2.4. Statistical approach and sample sizes

As our laboratory has recently observed male-female differences in hypoxia-induced dopamine cell dysfunction (unpublished findings), with males appearing to be more vulnerable than females, we used only male rat pups in this study. Power calculations that were based upon our preliminary studies suggested that no less than five male ω-3 PUFA enriched rats and five male


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control rats were required within each group to detect a mean minimum difference in dopamine levels of 25 ± 15%, with a power of 0.80 and α=0.05. Between group comparisons of dopamine levels were performed with an independent samples t-test, IBM SPSS version 20.

3. Results

To determine if maternal dietary supplementation with ω-3 PUFAs changed baseline dopamine levels, we measured striatal dopamine content within brain homogenates derived from PN 12 rat pups born to dams maintained on standard rat chow and compared them with striatal dopamine levels from ω-3 PUFA enriched rat pups. All pups were maintained in room air. Fig. 1 illustrates no difference in baseline striatal dopamine levels [mean ± 1 SEM picogram (pg) of dopamine per microgram (µg) of protein] between pups born to dams maintained on standard rat chow versus pups born to dams who received dietary supplementation with ω-3 PUFAs (25.83 ± 2.94 versus 29.87 ± 3.0 pg/µg protein, p=0.400).

To assess the extent that maternal dietary supplementation with ω-3 PUFAs may confer neuroprotection against hypoxic insults, we measured striatal dopamine content within rat pups (n=5) born to dams maintained on standard rat chow as well as within rat pups (n=9) born to dams who received ω-3 PUFA-enriched diets. Both groups were exposed to repetitive hypoxic insults between PN7-12. At PN 12, all pups were euthanized, brains were harvested, striatal tissue dissected, homogenized and analyzed with HPLC. Fig. 2 illustrates that striatal dopamine levels were significantly lower within post-hypoxic pups born to dams maintained on standard rat chow versus dopamine levels within post-hypoxic pups born to dams who received dietary supplementation with ω-3 PUFAs (17.99 ± 1.43 versus 33.29 ± 2.32 pg/µg protein, p=0.001).

![Dopamine pg /ug Protein](image)

**Fig. 1.** Striatal dopamine levels, as measured by HPLC, within PN12 rats born to dams maintained on either standard rat chow (grey bar) or ω-3 PUFA enriched rat chow (black bar). Diet did not influence dopamine levels; we found no inter-group difference. Maternal dietary supplementation with ω-3 PUFAs does not change striatal dopamine content.

**Fig. 2.** Striatal dopamine levels, as measured by HPLC, within PN12 rats born to dams maintained on standard rat chow or (grey bar) or ω-3 PUFA enriched rat chow (black bar). All pups were exposed to repetitive hypoxic insults between PN 7-12. Rat pups born to dams maintained on standard rat chow showed significantly reduced striatal dopamine levels when compared with the ω-3 PUFA enriched pups. Striatal dopamine is preserved within ω-3 PUFA enriched, post-hypoxic pups.

**Fig. 3.** Provides photomicrographs of substantia nigra pars compacta (SNpc) from two PN 12 rat pups exposed to hypoxia. Caspase-3 immunohistochemistry illustrates significant hypoxia-induced cell death (denoted by purple dots) in the rat maintained on the standard diet. Apoptosis was reduced in the ω-3 PUFA-enriched rat (right), which is consistent with preserved brain dopamine levels illustrated in Fig. 2.

4. Discussion

In this study, we found that ω-3 PUFA-enriched rat pups exposed to hypoxia between PN 7-12 experienced no loss of dopamine levels and SNpc apoptosis was diminished in (Fig. 3). In contrast, post-hypoxic control littermates born to dams maintained on normal diets experienced a 57% reduction in brain dopamine levels (Fig. 2) accompanied with significant apoptosis. These findings concur with a recent publication demonstrating neural resiliency against ischemic-anoxic insults in offspring of mothers who received ω-3 PUFA dietary supplementation during pregnancy [34].

Our findings, and those of Zhang [34] challenge the conclusions from the recent Cochrane Review of “Long chain polyunsaturated acid supplementation in infants born at term [35]”. That review summarized outcomes from multiple clinical trials assessing whether supplementing infant formula with ω-3 PUFA’s was safe and beneficial, to term infants. The author’s concluded that dietary supplementation with ω-3 PUFA’s was safe for infants but “Routine supplementation of milk formula with long chain polyunsaturated acids to improve the physical, neurodevelopmental or visual outcomes of infants born at term cannot be recommended based on the current evidence”.

The doses of ω-3 PUFA’s employed in clinical trials reviewed within the Cochrane report were well below the 3.5–4.0 g daily dose that we employed. Therefore, their conclusion that ω-3 PUFAs added to diets of healthy newborn children did not enhance
physical, neurodevelopment or visual outcomes is not surprising. It also could explain the divergence between other study findings that ω-3 PUFAs enriched diets could preserve physical, neurodevelopmental or visual integrity in rodent models of hypoxic, ischemic, anoxic events [36–39]. Despite those compelling findings, there have been no randomized controlled clinical trials to assess the efficacy of ω-3 PUFAs enriched diets for conferring neural resiliency against hypoxic insults in prematurely born children. Our preliminary studies suggest that prenatal dietary supplementation with ω-3 PUFAs do indeed confer neuroprotection against such adverse clinical events.

Our novel rodent model of maternal dietary supplementation enabled us to better control for environmental factors that we have frequently encountered during our human-based studies [40–42]. For example, our rodent model is not encumbered by the complexities associated with controlling for human dietary indiscretion that can negatively impact the assessment of nutritional supplements. In addition, the experimental flexibility provided by our rodent model enabled direct measurements of dopaminergic function and structure that cannot be readily performed in humans. We believe that by first defining the neurochemical outcomes conferred by ω-3 PUFA dietary supplementation in our rodent model, our future human-based protocols will be free to focus on hypothesis testing, rather than generation.

We selected to use 7–12 day old rat pups since seven day old rat’s cerebral cortex is comparable to humans born around 37 weeks (premature birth) [42]. By 12 days of age, the rat pup’s cortex is developmentally similar to a human born between 40 and 42 weeks gestation (term birth). We have previously shown that during this period of critical brain development, hypoxic insults induce significant impairment in brain dopaminergic systems [24,25]. Therefore, to be considered effective, we believe any putative neuroprotectant should confer resiliency to the dopaminergic system, against hypoxia, during this time period of vulnerability. Our findings suggest that ω-3 PUFAs may achieve this goal.

Another relevant feature of our model is the use of an iso-capnic, hypoxia-inducing gas mixture of 10% oxygen, 3–5% carbon dioxide, and balance nitrogen. This mixture evolved from our prior studies in both humans [41–45] and animals [46] employing both hypobaric and hypoxic challenges. During those studies we added 3.0–5.0% carbon dioxide (CO2) into the inspired gas mixture to provide an “atmospheric” pressure near 38 Torr. During hypoxic challenges, the addition of 3.0% supplemental inspired CO2 maintains the partial pressure of arterial CO2 (PaCO2) within the normative range of 38–45 mmHg. This prevents the acute reduction in PaCO2 that can occur during post-apnea hyperventilation (Fig. 4). Thus, during our protocol, pups become hypoxemic and hyperventilated, their inspired gas mixture sustained a PaCO2 within the upper end of the normative range. The net result was a relatively pure hypoxic insult without corresponding hypo- or hypercapnia, and other abrupt perturbations to acid–base physiology. We believe that minimizing potential blood acidosis (induced by CO2 buildup during apnea or anoxia) and alkalosis (induced by post-apnea hyperventilation), our gas mixture and hypoxia-inducing protocol provides insight into the pathogenic effect of hypoxia alone, and subsequently the neuroprotective effect of ω-3 PUFAs against hypoxia. Future experiments may be performed with gas mixtures designed to induce both hypoxia and hypercapnia, to further define the efficacy of ω-3 PUFAs against more severe apneic events.

Our selection of 10% oxygen within our gas mixture was informed by our prior studies in which we employed indwelling arterial catheters with simultaneous pulse oximetry to measure both partial pressures of arterial oxygen (PaO2) and hemoglobin oxygen saturation (SaO2) in rats exposed to hypobaric as well as hypoxic hypoxia [46]. Those studies revealed that a sustained inspired oxygen content of 10% produced a PaO2 of approximately 40 mmHg with a corresponding SaO2 of 75–80%. As Fig. 4 illustrates, an SaO2 near 80% is within the range observed during apneic events in a prematurely born infant at a gestational age of approximately 50 weeks at the time of the recording.

4.1. Limitations and future studies

We acknowledge that our study design cannot provide insight into whether putative ω-3 PUFA conferred neuroprotection originated within the fetal brain prior to birth via ω-3 PUFA’s conveyed in fetal circulation, or following birth via ω-3 PUFA’s conveyed in mother’s colostrum [47,48]. If neuroprotection occurred prior to birth, this may suggest that ω-PUFA’s were conveyed directly through placental circulation. If neuroprotection occurred post-birth, this may suggest that PUFA’s were conveyed through the

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**Fig. 3.** Photomicrographs of the substantia nigra pars compacta (SNpc) from two PN 12 rat pups exposed to hypoxia. The SNpc from the pup maintained on a standard diet is on the left. The SNpc from the pup maintained on a ω-3 PUFA-enriched diet is on the right. Each small circular black dot identifies a caspase-3 positive cell undergoing apoptosis. Apoptosis is reduced in the ω-3 PUFA-enriched rat (right). ω-3 PUFAs reduce hypoxia-induced apoptosis.
digestive tract. In addition, future studies are also needed to characterize the relationship between the dietary content of consumed ω-PUFA’s with subsequent circulatory and neuronal levels.

Our study was not designed to uncover the potential mechanism through which ω-3 PUFA’s conferred their beneficial effects. Recent observation by Chang et al. [49] suggest that the DHA component of ω-3 PUFA’s suppress post-ischemia neural damage by suppressing release of proinflammatory cytokines. Oxidative damage was concomitantly attenuated by reducing c-Jun N-terminal kinase (JNK) phosphorylation and activating protein (AP-1) signaling. Future studies are needed to determine if these same mechanisms contributed to the neuroprotection of the dopaminergic system that we observed in ω-3 PUFA enriched newborns pups exposed to hypoxic insults.

5. Conclusions

In spite of this study’s potential limitations, our novel findings suggest that it may be feasible to confer some degree of neuroprotection to newborns at risk for hypoxic insults. Our future studies are directed towards characterizing the extent that behavioral, motor and cognitive function is also preserved in ω-3 PUFA enriched newborns exposed to those insults. Collectively, those and other studies are necessary to confirm and extend findings presented here. Doing so may also provide the scientific rationale to begin considering the feasibility to shift current clinical paradigms away from focusing only upon maximizing the remaining function of the post-hypoxic brain, to proactively conferring neuroprotection against such insults.

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