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Regional differences of hypothermia on oxidative stress following hypoxia-ischemia: a study of DHA and hypothermia on brain lipid peroxidation in newborn piglets

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Abstract

Background: Oxidative stress plays an important part in the pathophysiology of hypoxic-ischemic encephalopathy (HIE) and is reliably measured through prostanoids following lipid peroxidation of polyunsaturated fatty acids (PUFAs). The aim of the study is to measure oxidative stress in the prefrontal cortex, white matter and hippocampus in the brains of hypoxic-ischemic piglets treated with docosahexaenoic acid (DHA) and therapeutic hypothermia (TH) and investigate the additive effects of DHA on hypothermia by factorial design.

Methods: Fifty-five piglets were randomized as having severe global hypoxia (n=48) or not (sham, n=7). Hypoxic piglets were further randomized: vehicle (VEH), DHA, VEH+hypothermia (HT) or HT+DHA. A total of 5 mg/kg DHA was given intravenously 210 min after the end of hypoxia. Brain tissues were analyzed using liquid chromatography triple quadrupole mass spectrometry technique (LC-MS). A two-way analysis of variance (ANOVA) was performed with DHA and HT as main effects.

Results: In the white matter, we found main effects of DHA on DH-isoprostanes (P=0.030) and a main effect of HT on F₄-neuroprostanes (F₄-NeuroPs) (P=0.007), F₂-isoprostanes (F₂-IsoPs) (P=0.043) and DH-isoprostanes (P=0.023). In

the cortex, the ANOVA analysis showed the interactions of main effects between DHA and HT for neurofuranes (NeuroFs) (P=0.092) and DH-isoprostanes (P=0.015) as DHA significantly reduced lipid peroxidation in the absence of HT. DHA compared to VEH significantly reduced NeuroFs (P=0.019) and DH-isoprostanes (P=0.010). No differences were found in the hippocampus.

Conclusion: After severe hypoxia, HT reduced lipid peroxidation in the white matter but not in the cortical gray matter. HT attenuated the reducing effect of DHA on lipid peroxidation in the cortex. Further studies are needed to determine whether DHA can be an effective add-on therapy for TH.

Keywords: DHA; isoprostanes; lipid peroxidation; neuroprostanes; oxidative stress; therapeutic hypothermia; white matter injury.

Introduction

Hypoxic-ischemic encephalopathy (HIE) affects 1.5 per 1000 newborns in industrialized countries and the pathophysiology encompasses a primary and secondary energy failure where oxidative stress and reactive oxygen species play an important part on the latter [1, 2]. Therapeutic hypothermia (TH) has become the standard of care for moderate and severe HIE, but because the pathogenesis of HIE involves multiple post-ischemic cascades leading to cell death, effective treatment of ischemic brain injury is likely to require intervention at multiple effect sites like anti-inflammatory, anti-apoptotic and anti-oxidative treatment.

Docosahexaenoic acid (DHA) is an Ω -3 polyunsaturated fatty acid (PUFA) highly expressed in the developing brain and contributing more than 30% of the entire phospholipid membrane content [3]. The Ω -6 PUFA arachidonic acid is abundant in myelin and white matter. When exposed to free radicals from ROS production PUFAs are nonenzymatically peroxidized to prostanoids, which are prostaglandin-like compounds. Arachidonic acid (AA) is peroxidized to F₂-isoprostanes (F₂-IsoPs), DHA to F₄-neuroprostanes (F₄-NeuroPs) or neurofuranes (NeuroFs) and

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adrenic acid (AdA) to di-homo-isoprostanes (DH-IsoPs) [4, 5]. The F_2 -IsoPs are considered as some of the most reliable markers of oxidative stress in humans [6]. AA is evenly distributed in the brain, whereas DHA and AdA are mainly confined to the neurons and white matter, respectively, and hence the prostanoids are region-specific markers of damage [7]. The liquid chromatography triple quadrupole mass spectrometry (LC-MS) analysis is a sensitive and specific method for quantifying prostanoids which strengthen their position as biomarkers of *in vivo* oxidative stress [8].

DHA has been used therapeutically and acts as a neuroprotectant following traumatic brain and spinal cord injuries in adults [9] and augments TH in a rodent model of newborn hypoxia-ischemia where rats treated with therapeutic hypothermia combined with DHA had reduced brain loss after hypoxia-ischemia compared to control animals [10]. We recently published how DHA significantly reduced F_4 -NeuroPs and the isoprostane compound 8-iso-PGF_{2 α} in urine after severe hypoxia [11]. Histopathology showed that there was a significant difference between hypoxic and normoxic piglets, but there were no differences between the intervention groups.

Here, we present the results from brain tissues. We wanted to confirm our previous findings of reduced lipid peroxidation in the prefrontal cortex and hippocampus of DHA-treated piglets [12] and further expand this model to include tissue from the white matter and also include treatment with hypothermia (HT). We hypothesize that both DHA and HT will reduce brain lipid peroxidation and that the treatments will positively interact.

Materials and methods

Approval

The Norwegian Council for Animal Research approved the experimental protocol, No 5723. The animals were cared for and handled in accordance with the European Guidelines for Use of Experimental Animals, by certified Federation of European Laboratory Animal Science Associations (FELASA) Category C researchers.

Experimental design

The current study is one half of a larger experimental design of 81 newborn Noroc (LyxLD) pigs randomized to interventions of DHA, cannabidiol (CBD) or TH. The two studies have three shared groups [vehicle (VEH), HT and sham] in order to spare animal lives. The CBD part of the study has been published [13]. The complete experimental design is presented in Supplementary Material, Figure S1. The piglets had inclusion criteria of 12–36 h of age, Hb >5 g/dL and good general

condition. Exclusion criteria were a hemoglobin level below 5 g/dL, signs of sickness or protocol breach.

Fifty-five piglets were part of the present study. Forty-eight of these were subjected to experimental interventions. A sham group of seven piglets did not receive hypoxia. The methods are have been described previously in detail [13]. Briefly, piglets were anesthetized, intubated and kept on mechanical ventilation throughout the experiment. The left jugular vein and right carotid artery were cannulated for venous access and hemodynamic monitoring. Hypoxia was induced by ventilation with 8% O₂ until base excess –20 mmol/L or mean arterial blood pressure (MABP) <20 mm Hg before resuscitation with 21% O₂. After reoxygenation, the piglets were randomized to one of four intervention groups: (i) VEH (n = 12), (ii) DHA (n = 12), (iii) VEH + HT (n = 12) and (iv) HT + DHA, (n = 12).

DHA (1 g cis-4, 7, 10, 13, 16, 19-DHA) was dissolved in 0.9% NaCl and diluted to 10 mg/mL. Piglets randomized to DHA received 5 mg/kg intravenously 3.5 h after the end of hypoxia. Piglets randomized to TH were cooled with a cooling mattress (Tecotherms TSmed 200; TecCo, Halle, Germany) with a target rectal temperature of 34.5 °C. All the other piglets were maintained normothermic at 39 °C (±0.5). The piglets were euthanized with 150 mg/kg intravenous pentobarbital, 9.5 h after end of hypoxia.

LC-MS

The brain was immediately removed, and samples from the prefrontal cortex and subcortical white matter tracts of the cerebrum were harvested and snap frozen in liquid nitrogen before storage at –80 °C. The hippocampus was extracted as a whole and 1/3 was sent for analysis. A total of 100 mg of frozen cortical, hippocampal and white matter tissue was prepared by methods previously described by Casetta et al. and was subjected to liquid chromatography-mass spectrometry (LC-MS) [12, 14]. All samples were blinded prior to the analysis.

Statistics

Statistics were done using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 23 (SPSS, Chicago, IL, USA). The physiological data were normally distributed and are expressed as mean ± standard deviation (SD). Shapiro-Wilk's test was applied to test for normality on the lipid peroxidation data, and log-transformation was performed on non-normally distributed data. If log-transformation did not produce a normal distribution, non-parametric statistics were applied. Lipid peroxidation measurements were tested using a two-way analysis of variance (ANOVA) with HT and DHA as main factors, meaning groups treated with DHA were DHA and DHA + HT and groups treated with HT were VEH + HT and HT + DHA. The reciprocal groups were non-DHA (VEH and HT) and non-hypothermia (VEH and DHA). Sham piglets are not included in the main model. First, we tested for interactions between the two main factors, if found significant at $P \leq 0.10$ *post hoc* two-by-two group comparisons were made. P-values were adjusted by Fisher's least significant difference (LSD) *post hoc* test. However, if interactions were not present, only main effects were maintained in the model.

Data for the experiments are displayed as box and whisker plots with maximum, 75th percentile, median, 25th percentile and minimum. P-values <0.05 were considered statistically significant and given together with a 95% confidence interval of the mean difference.

Results

Animals and groups

One pig in the DHA group was eliminated from the study upon autopsy due to massive cerebral edema making the brain impossible to harvest. Physiology of the groups is shown in Table 1. The target hypothermic temperature was attained after 105 min in the VEH + HT group and after 96 min in the DHA + HT group.

Lipid peroxidation

Table 2 shows descriptive statistics and P-values of the main effects. Untransformed mean $\pm$ SD values for the randomized groups are shown in Supplementary Material, Table S1.

Cortex

One extreme outlier in the group HT + DHA was excluded (sample value of DH-IsoP = 120 ng/g, 5 SD above mean).

The two-way ANOVA analysis revealed interactions for NeuroFs ($P = 0.092$) and DH-IsoPs ($P = 0.015$) (values shown in Table 2, profile plots in Supplementary Material, Figure S2). A separate one-way ANOVA analysis was then performed on the randomized groups. NeuroFs and DH-IsoPs were significantly reduced in DHA-treated animals compared to VEH when HT was not present (Figure 1, values in Supplementary Material, Table S1): NeuroFs (mean $\pm$ SD: 0.19 ± 0.18 vs. 0.33 ± 0.11 log ng/g; $P = 0.019$) and DH-IsoPs (28.8 ± 13.9 vs. 45.1 ± 13.3 ng/g; $P = 0.010$). No effect of HT was found on the cortical gray matter.

White matter

There were no interactions between DHA and HT (Table 2). There were significant main effects of HT on F_2 -IsoPs (HT vs. non-HT: 7.5 ± 0.81 vs. 9.9 ± 0.86 log ng/g; $P = 0.043$), F_4 -NeuroPs (240 ± 3.7 vs. 463 ± 91.3 ; $P = 0.007$) and DH-IsoPs (105 ± 10.1 vs. 144 ± 13.9 ng/g; $P = 0.023$). DHA had a main effect on DH-IsoPs (DHA vs. non-DHA: 105 ± 9.1 vs. 143 ± 14.4 ng/g; $P = 0.030$) and tended to be associated with reduced levels of F_4 -NeuroPs, but did not reach a significance level of $P < 0.05$ (Figure 2).

Hippocampus

No interactions or significant main effects were found in the hippocampal tissues.

Discussion

The present study shows how HT and DHA significantly reduced lipid peroxidation in the white matter, but we could not find a positive interaction effect. In the cortical tissue, HT did not reduce NeuroFs and DH-IsoPs when combined with DHA. We could only partly confirm our previous findings of significantly reduced lipid peroxidation in the prefrontal cortex of piglets treated with DHA, given the absence of HT.

Hypothermia

We recently published results from lipid peroxidation in urine of the same hypoxic piglets as in the present paper, where we found that HT reduced 8-Iso-PGF_{2 α} , but none of the other measured compounds [11]. In the results from the brain tissue we found that TH had a main effect in the white matter where it significantly reduced lipid peroxidation in three of the four measured compounds. Interestingly, HT seemed to inhibit DHA's reduction on lipid peroxidation in the cortical tissue at 9.5 h after end hypoxia. This may be due to a negative interaction or the result of small numbers in the study. In addition, it is not known whether lipid peroxidation would increase or decrease towards 48 h post hypoxia. Van Rollins et al. [5] have shown isoprostanes to peak at 16 h after exposure to oxidative stress in the gray matter and still be increasing after 24 h in the white matter. They did not investigate the relationship between HT and lipid peroxidation, nor did they measure at 48 h after hypoxia.

The effect of HT on lipid peroxidation is not fully established in the literature. Some small studies have shown that HT in fact increases lipid peroxidation [15] and this was also the conclusion of a review article by Alva et al. [16] but methods of measuring lipid peroxidation was different from ours. On the contrary, Hasegawa et al. [17] showed how cooling PC12 cells to 32 °C again reduced lipid peroxidation. In concordance with our findings, Bayir et al. [18] found no effect of HT on lipid peroxidation in the cortex after traumatic brain injuries in adults. In the same study, hypothermic males but not females had increased F_2 -IsoPs in CSF and it was advised to exhibit caution in

Table 1: Physiology of the intervention groups throughout the experiment.

Measurements	VEH	DHA	VEH + HT	DHA + HT	Sham	P-value
Body weight, g	1970 (100)	2012 (136)	2006 (118)	2055 (97)	2028 (24)	0.43
Sex (male:female)	1.4	1.4	0.5	0.5	0.75	0.55
Post-natal age, h	25.7 (2.6)	26.7 (5.1)	26.8 (3.3)	25.3 (4.2)	25.3 (2.8)	0.79
Hypoxia time, min	41.5 (10)	42.1 (18)	41.4 (10)	41.4 (17)	0.0	1.0
Cooling time (min of 34.5 °C)	0	0	465	474	0	0.00
Hemoglobin, g/dL	7.5 (1.1)	7.3 (1.4)	7.8 (1.4)	7.3 (1.3)	7.8 (1.0)	0.87
Base excess, mmol/L						
Start hypoxia	1.7 (2.3)	2.0 (3.0)	1.3 (3.4)	2.5 (3.0)	0.5 (2.7)	0.65
End hypoxia	-20.2 (1.2)	-20.3 (0.4)	-20.0 (0.5)	-19.5 (3.2)	0.3 (1.4)	0.00
Reox210	0.6 (2.5)	-0.2 (2.7)	-0.1 (2.5)	0.5 (2.2)	1.9 (2.4)	0.017
End study	1.7 (2.9)	-1.1 (3.5)	-2.8 (3.8)	0.3 (3.0)	-0.3 (3.5)	0.046
Lactate, mmol/L						
Start hypoxia	2.2 (0.8)	2.025 (0.6)	1.9 (0.6)	2.0 (0.8)	2.4 (1.0)	0.70
End hypoxia	15.7 (2.5)	16.9 (2.9)	16.2 (1.9)	15.7 (2.3)	1.7 (0.4)	0.00
Reox210	1.9 (0.8)	2.0 (1.6)	1.8 (0.7)	2.0 (1.5)	1.4 (0.3)	0.78
End study	1.3 (0.4)	1.5 (2.0)	2.0 (1.6)	1.3 (0.4)	1.1 (0.3)	0.48
Temperature, °C						
Start hypoxia	39.3 (0.7)	39.3 (0.7)	39.3 (0.5)	39.4 (0.8)	39.6 (0.6)	0.90
End hypoxia	38.6 (0.5)	38.4 (0.5)	38.2 (0.5)	38.5 (0.3)	39.5 (0.8)	0.000
Reox210	39.5 (0.7)	39.0 (0.6)	35.1 (0.4)	35.2 (0.7)	39.6 (0.7)	0.000
End study	39.2 (0.6)	38.9 (0.8)	35.0 (0.5)	35.1 (0.4)	39.1 (0.5)	0.000
Mean arterial blood pressure, mm Hg						
Start hypoxia	65.4 (11.4)	62.6 (11.5)	68.5 (7.5)	60.5 (9.7)	69.3 (10.8)	0.33
End hypoxia	43.1 (18.4)	47.1 (21.4)	39.6 (18.4)	48.3 (24.7)	71.4 (13.9)	0.017
Reox210	58.3 (10.9)	54.5 (9.1)	61.9 (10.4)	53.4 (10.5)	59.6 (7.5)	0.44
End study	56.3 (7.5)	51.3 (13.1)	49.9 (9.7)	51.6 (13.0)	56.1 (3.6)	0.49
pO ₂ , kPa						
Start hypoxia	9.3 (0.22)	9.77 (0.56)	9.36 (0.68)	10.29 (0.37)	9.65 (0.40)	0.59
End hypoxia	4.69 (0.99)	4.32 (0.5)	4.67 (0.5)	4.46 (0.59)	10.6 (1.3)	0.001
Reox210	9.9 (1.0)	10.3 (1.9)	8.4 (0.86)	8.9 (1.4)	10.4 (1.2)	0.003
End study	9.9 (1.1)	9.4 (1.3)	9.4 (1.5)	9.8 (1.9)	10.5 (1.9)	0.51
pCO ₂ , kPa						
Start hypoxia	5.14 (0.71)	5.7 (2.0)	5.6 (0.6)	5.2 (0.6)	5.6 (0.6)	0.59
End hypoxia	8.8 (1.4)	9.3 (1.3)	9.2 (0.8)	9.2 (1.3)	5.5 (1.9)	0.000
Reox210	5.0 (0.8)	4.3 (0.7)	5.7 (0.7)	4.7 (0.5)	4.8 (0.7)	0.000
End study	4.5 (0.6)	4.9 (0.6)	5.1 (0.9)	4.5 (0.6)	4.7 (0.5)	0.088
Glucose, mmol/L						
Start hypoxia	7.0 (1.4)	7.4 (1.1)	7.5 (1.8)	6.7 (1.6)	7.5 (0.9)	0.61
End hypoxia	11.1 (5.3)	10.3 (3.5)	11.5 (5.0)	10.2 (5.8)	6.6 (1.1)	0.25
Reox210	5.7 (1.6)	5.5 (1.1)	5.5 (0.9)	5.5 (2.3)	5.7 (0.6)	0.99
End study	5.1 (1.0)	5.5 (0.9)	5.6 (1.1)	5.3 (1.3)	5.3 (0.7)	0.86
pH						
Start hypoxia	7.44 (0.05)	7.44 (0.06)	7.4 (0.06)	7.44 (0.06)	7.4 (0.06)	0.11
End hypoxia	6.86 (0.05)	6.84 (0.04)	6.85 (0.03)	6.86 (0.08)	7.43 (0.04)	0.00
Reox210	7.43 (0.07)	7.47 (0.08)	7.38 (0.06)	7.45 (0.06)	7.47 (0.08)	0.017
End study	7.43 (0.07)	7.41 (0.08)	7.38 (0.09)	7.46 (0.06)	7.44 (0.04)	0.046
HR, bpm						
Start hypoxia	177 (52)	168 (30)	153 (28)	163 (28)	152 (35)	0.50
End hypoxia	190 (34)	173 (25)	185 (52)	188 (27)	158 (40)	0.35
Reox210	214 (50)	208 (66)	163 (29)	178 (37)	162 (31)	0.027
End study	197 (53)	208 (53)	144 (30)	170 (46)	168 (42)	0.019

Values listed as mean ($\pm$ SD). Significant group differences (one-way ANOVA): P-values in bold. The groups were comparable as there were no intergroup differences in body weight, post-natal age, hypoxia time or baseline physiological (heart rate, mean arterial blood pressure) and biochemical measures (blood lactate, base excess and glucose).

Table 2: Descriptive statistics of pooled groups and P-values of main effects.

	DHA	Non-DHA	HT	Non-HT	ANOVA	Main DHA	Hypothermia effects
Cortex							
F ₂ -IsoPs ^a	3.9 (.29)	4.4 (.28)	4.1 (.24)	4.2 (.34)	0.25	0.106	0.74
F ₄ -NeuroPs	135 (16.5)	165 (17.9)	148 (16.2)	153 (18.9)	0.31	0.24	0.85
NeuroFs ^a	7.7 (1.1)	9.8 (1.2)	9.5 (1.3)	8.1 (.92)	0.092^b	—	—
DH-IsoPs	38.4 (4.9)	40.6 (2.9)	41.7 (4.6)	37.3 (3.3)	0.015^b	—	—
White matter							
F ₂ -IsoPs ^a	8.7 (0.97)	8.6 (0.78)	7.5 (0.81)	9.9 (0.86)	0.69	0.85	0.043
F ₄ -NeuroPs ^a	267 (28)	428 (89)	240 (23.7)	463 (91.3)	0.69	0.087	0.007
NeuroFs ^a	17.1 (2.3)	23.6 (3.1)	18.6 (2.9)	22.3 (2.6)	0.32	0.094	0.11
DH-IsoPs	105 (9.1)	143 (14.4)	105 (10.1)	144 (13.9)	0.78	0.030	0.023
Hippocampus							
F ₂ -IsoPs	5.0 (.62)	5.2 (.71)	5.1 (.66)	5.1 (.69)	0.91	0.87	0.99
F ₄ -NeuroPs	322 (68.3)	336 (82.8)	372 (85.9)	284 (62.5)	0.63	0.98	0.37
NeuroFs	11.5 (2.0)	11.8 (2.1)	12.6 (2.2)	10.6 (1.9)	0.23	0.91	0.50
DH-IsoPs	86.2 (16.7)	86.1 (15.3)	90.9 (16.7)	81.2 (15.0)	0.64	0.99	0.67

Values given as mean (standard error). Pooled groups are: DHA, DHA and DHA + HT (n = 23); non-DHA, VEH and HT (n = 24); HT, VEH + HT and HT + DHA (n = 24); non-HT, VEH and DHA (n = 23). ^aANOVA analysis performed on log-transformed data. Significant findings in bold. ^bThe overall two-way ANOVA interaction test used a somewhat significance level of $P < 0.10$ to allow a lower threshold for detecting interactions and $P < 0.05$ for main effects. NeuroFs and DH-IsoPs in the cortex show significant interactions and a one-way ANOVA with LSD *post hoc* tests were performed on data from the randomized groups (values in text, not shown in table).

Figure 1: Prostanoids in the cortex.

Non-parametric data distributions are converted and displayed as log-transformations to enable parametric analysis. The bottom two figures of NeuroFs and DH-IsoPs showed interactions in the overall ANOVA analysis ($P < 0.10$). Further one-way ANOVA was done on the randomized groups as shown in the figure. * $P < 0.05$, ** $P < 0.01$.

Figure 2: Prostanoids in the white matter.

No interactions were found in the white matter. Main effects of DHA and hypothermia on lipid peroxidation products as shown in figure.

* $P < 0.05$, ** $P < 0.01$.

combining anti-oxidant treatment with TH. We found no gender effect in our study.

Hippocampus

Belayev et al. [19] have shown DHA to be neuroprotective and have treatment potential following brain ischemia in rats. We recently published how DHA reduces F₂-IsoPs and F₄-NeuroPs in the cortex and F₂-IsoPs and DH-IsoPs in hippocampus [12]. The present study partly supports these findings of reduced prostanooids in the cortex. We found no differences in the hippocampus.

There is region-specific vulnerability to lipid peroxidation and oxidative stress in the human brain [20]. In the hippocampus, the majority of neurons are densely packed into a single layer divided into regions CA1 through CA4. Despite the physical proximity and cell morphological similarity, CA1 and CA3 neurons respond to oxidative stress very differently. Wilde et al. [21] found that the pyramidal neurons in the CA1 region suffer massive cell death by 64% while in CA3 there is only 6% cell death

when exposed to oxidative stress-generating agents like superoxide. In a newborn piglet study, CA1 and the dentate gyrus were the regions that were most vulnerable to hypoxia-ischemia [22]. In the present study, we did not standardize which areas of the hippocampus to analyze, and this may be the reason no differences were found.

White matter

White matter of the developing brain is especially vulnerable to hypoxia-ischemia. Ikeda et al. [23] showed how lipid peroxidation displayed regional distribution as the white matter was significantly more susceptible to damage than the gray matter. They have also shown that thiobarbituric acid reactive substances in the white matter correlate with morphological changes 72 h post hypoxia in fetal lambs.

White matter injuries are prominent in both preterm and full-term asphyxiated infants. Back et al. [24] found increased F₂-IsoPs as a marker of early periventricular white matter injury and early HIE. Recent developments

in lipidomics have shown DH-isoprostanes to be an even more sensitive marker of the white matter injury [25]. Preterm infants are at additional risk of damage from oxidative stress as they have low antioxidant defenses and the transplacental surge of DHA does not occur until the last trimester. A 1% increase in postnatal DHA-levels gave a four-fold reduction of intraventricular hemorrhage in premature infants [26]. Premature infants may hence benefit from DHA supplementation to combat intraventricular hemorrhage and improve microstructural brain development.

Dietary supplementation of DHA has reduced lipid peroxidation and decreased white matter injury on MRI screening in rat pups subjected to controlled cortical impact [27]. We found DHA to have a main effect on reducing DH-IsoPs, the major PUFA in the white matter.

DHA

To our knowledge, ours is the first study to investigate DHA and TH in a piglet model of hypoxia-ischemia. We found that DHA reduced DH-IsoPs in the white matter and that it reduced NeuroFs and DH-IsoPs in the cortical gray matter in the absence of HT. These results support the neuroprotective findings of Berman et al. [10] on hypothermic DHA-supplemented rats. But we could not, unlike Berman, find a positive interaction between DHA and HT at this early time point after end hypoxia. TH requires intensive care facilities and is the only established treatment for HIE. Worldwide, a majority of children are not born in a hospital with a neonatal intensive care unit. There is mounting evidence supporting the beneficial effects of DHA on neurodegenerative and neurological conditions [28]. DHA accumulates in the brain starting *in utero* through toddlerhood coinciding with the extensive brain development in this period [29]. Berman et al. [30, 31] have shown how both pre-hypoxic and post-hypoxic treatments with DHA in a neonatal rat model improve functional outcome on forepaw placing. Our results show how DHA selectively reduces oxidative stress in the normothermic gray matter and white matter in the piglet brain. Future studies should explore DHA's potential as a neuroprotective drug after hypoxia-ischemia outside the neonatal intensive care unit.

Strengths and limitations

Our model inflicts severe hypoxia-ischemia by biochemical measures and MABP, but does not provide EEG

readings. The model is supported by Domoki et al.'s [32] recommendations of utilizing hypoxic ventilation with systemic hypotension to reproduce the ischemic aspect of HIE in piglets.

The model is a newborn model of asphyxia as piglets have gone through perinatal transition. The dosage and timing of DHA administration are based on previous rat studies and it is not studied whether this is optimal in piglets, but delayed timing of DHA administration is clinically relevant.

The model does not include rewarming, and the metabolic effects from HT and the subsequent rewarming process have not been investigated.

Due to few piglets in each group and multiple comparisons, we chose an α -level ≤ 0.10 for the interaction level of the two-way ANOVA. This was to allow for more subtle interactions to be revealed, as they are more difficult to detect than main effects which the study was originally intended for.

Our model has a short follow-up time of 9.5 h post end hypoxia limiting the possibility to assess neuroprotection by DHA. The model gives insight to processes occurring in the latent and early second-injury phase of the HIE development with high levels of oxidative stress.

Conclusion

We found reduced oxidative stress in both prefrontal cortex and white matter (but not in the hippocampus) in piglets treated with DHA, but we could not find any additive effect of HT on DHA. HT exerted regional differences on lipid peroxidation; as in the white matter it reduced oxidative stress, but in the cortex HT negated DHA's reducing effect on lipid peroxidation. Further studies are needed to determine DHA's potential neuroprotective properties with and without TH.

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