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DHA reduces oxidative stress following hypoxia-ischemia in newborn piglets: a study of lipid peroxidation products in urine and plasma

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Abstract

Background: Lipid peroxidation mediated by reactive oxygen species is a major contributor to oxidative stress. Docosahexaenoic acid (DHA) has anti-oxidant and neuroprotective properties. Our objective was to assess how oxidative stress measured by lipid peroxidation was modified by DHA in a newborn piglet model of hypoxia-ischemia (HI).

Methods: Fifty-five piglets were randomized to (i) hypoxia, (ii) DHA, (iii) hypothermia, (iv) hypothermia + DHA or (v) sham. All groups but sham were subjected to hypoxia by breathing 8% O_2. DHA was administered 210 min after end of hypoxia and the piglets were euthanized 9.5 h after end of hypoxia. Urine and blood were harvested at these two time points and analyzed for F_4-neuroprostanes, F_2-isoprostanes, neurofuranes and isofuranes using UPLC-MS/MS.

Results: F_4-neuroprostanes in urine were significantly reduced ($P = 0.006$) in groups receiving DHA. Hypoxia (median, IQR 1652 nM, 610–4557) vs. DHA (440 nM, 367–738, $P = 0.016$) and hypothermia (median, IQR 1338 nM, 744–3085) vs. hypothermia + DHA (356 nM, 264–1180, $P = 0.006$). The isoprostane compound 8-iso-PGF2α was significantly lower ($P = 0.011$) in the DHA group compared to the hypoxia group. No significant differences were found between the groups in blood.

Conclusion: DHA significantly reduces oxidative stress by measures of lipid peroxidation following HI in both normothermic and hypothermic piglets.

Keywords: Docosahexaenoic acid (DHA); hypoxia-ischemia (HI); isoprostanes; lipid peroxidation; neuroprostanes; neuroprotection; oxidative stress; reactive oxygen species.

Introduction

During perinatal asphyxia, the body is deprived of oxygen and glucose, initiating a cascade of harmful processes including necrosis, apoptosis, production of reactive oxygen species (ROS) and lipid peroxidation [1]. These processes can lead to the clinical condition of hypoxic-ischemic encephalopathy (HIE) [2]. ROS-mediated lipid peroxidation is especially important in the brain due to its high content of lipids. Polyunsaturated fatty acids (PUFAs) are highly susceptible to lipid peroxidation through oxidative stress because of their high content of double bonds. When major PUFAs like arachidonic acid and docosahexaenoic acid (DHA) are oxidized, they can be measured in different tissue fluids through their downstream metabolites F_2-isoprostanes, F_4-neuroprostanes, isofuranes and neurofurane [3]. These metabolites may be measured though rigorous ultra performance liquid chromatography- tandem mass spectrometry (UPLC-MS/MS) [4].

F_2-isoprostanes are products of non-enzymatic free radical catalyzed oxidation of arachidonic acid and they are currently thought to be the most reliable markers of oxidative damage in humans [5]. Welin et al. [6] showed how F_2-isoprostane increased in plasma, hours after

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umbilical cord clamping in mid-gestation fetal sheep and how the lack of increase in the melatonin treated group correlated to reduced white matter injury. Recently, Chafer-Pericas et al. [7] published a study on how the levels of total F_2-isoprostanes and the compound 8-iso-15(R)-$PGF_{2\alpha}$ correlate with the severity of post-natal acidemia, suggesting a new potential for these metabolites as biomarkers of HIE.

F_4-neuroprostanes are formed from lipid peroxidation of DHA and are considered sensitive and specific markers of neuronal oxidative damage [8].

DHA is the most abundant n-3 fatty acid in cell membranes and contributes to more than 30% of the entire phospholipid content in neural membranes [9]. DHA is present in all organs but the concentration varies greatly from organ to organ. Arterburn et al. [10] have presented distribution patterns of four PUFAs in different organs in adults. The retina (22 g/100 g of total fatty acid) has the highest presence of DHA, followed by sperm (14 g/100 g total fatty acid) and the cerebral cortex (13 g/100 g of total fatty acid). A fetus requires especially large amounts of DHA during the last trimester and in the first 2 years of life, coinciding with extensive brain development during this period [11]. The immature brain is especially sensitive to oxidative damage in comparison to the mature brain because it has poor antioxidant capabilities and a high concentration of free iron and lipids.

Ingested DHA has improved neurogenesis and neuroplasticity [12] in adult hippocampal tissue. Hong et al. [13] found a significantly reduced cerebral infarction volume in rats supplemented with DHA following middle cerebral artery occlusion. It has also been shown that a maternal diet rich in DHA can improve motor skills during infancy [14] and improve the child's IQ at 4 years of age [15].

Previously published papers on experimental neonatal hypoxia-ischemia (HI) and treatment with therapeutic hypothermia and DHA for neuroprotection have, to our knowledge, been based on the use of rodents [16, 17]. In this experimental piglet model, we wanted to test through lipid peroxidation the hypothesis that DHA reduces oxidative stress following HI and that DHA also reduces oxidative stress during therapeutic hypothermia, the established treatment for HIE. Non-invasive techniques are important in neonatal practice and hence we primarily wanted to investigate the outcome of lipid peroxidation in urine and also compare the urine samples to plasma samples. DHA peroxidation metabolites in urine and plasma reflect DHA distribution patterns, predominantly reflecting oxidative stress in the brain.

Methods

Approval

The Norwegian Council for Animal Research approved the experimental protocol. The animals were cared for and handled in accordance with the European Guidelines for Use of Experimental Animals, by certified FELASA (Federation of European Laboratory Animals Science Associations) Category C researchers with approval number 5723.

Set-up

Fifty-five newborn Noroc (LyxLD) pigs were included in the study, with the inclusion criteria of 12–36 h of age, Hb > 5 g/dL and healthy appearance. Forty-eight of these were subjected to experimental interventions. A sham group of seven piglets went through the same experimental set-up (were anesthetized, sham operated and ventilated) but were not subjected to hypoxia and reoxygenation. The study had the exclusion criteria of Hb < 5 g/dL, poor general condition or protocol breech.

Surgical preparation and anesthesia

The details of surgery and anesthesia are previously described in detail [18]. Briefly, piglets were initially anesthetized with i.v. pentobarbital, fentanyl and midazolam and orally intubated prior to surgical cannulation of the left jugular vein and right carotid artery for invasive, continuous monitoring of the mean arterial blood pressure (MABP), heart rate and rhythm. The inspired fraction of O_2 was 0.21 throughout the entire experiment, except during hypoxia. Rectal temperature was maintained between 38.5°C and 39.5°C with a heating blanket. The piglets were euthanized with 150 mg/kg of pentobarbital, given intravenously, 9.5 h after end of hypoxia.

Experimental protocol

The piglets were block randomized twice. The operators were not blinded to the randomization. The first randomization yielded seven piglets for sham operation and the remaining 48 underwent hypoxia. The experimental protocol is summarized in Figure 1. Further details and maintenance are as previously described [18]. The second randomization occurred immediately following hypoxia and the piglets were assigned to one out of four intervention groups: (i) hypoxia (ii) DHA (iii) hypothermia and (iv) hypothermia + DHA. Hypothermia was induced within 15 min after end of hypoxia and lasted to end of study. Target rectal temperature was 34.5 °C. Hypothermia was achieved by turning off all heat and using a cooling blanket to regulate the temperature. DHA (1 g CIS-4, 7, 10, 13, 16, 19-docosahexaenoic acid) was dissolved in 0.9% NaCl and diluted to 10 mg/mL. The piglets were given 5 mg/kg i.v. at 210 min (3.5 h) after end of hypoxia.

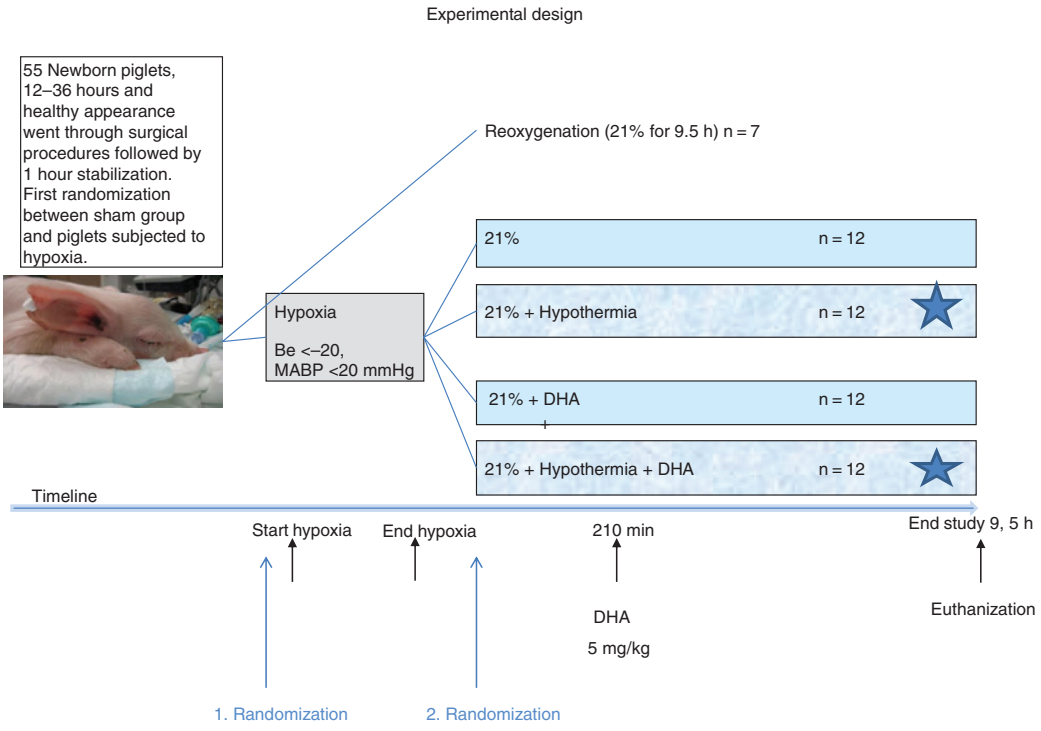

Figure 1: Experimental design.

Plasma and urine samples

Temperature-corrected arterial acid/base status, glucose and hemoglobin were regularly measured throughout the experiment on a Blood Gas Analyzer 860 (Ciba Corning Diagnostics, Medfield, MA, USA). Plasma samples were drawn before initiating hypoxia and at end of hypoxia. After reoxygenation, plasma samples were drawn at 30 min and 210 min after end of hypoxia before the final samples were drawn at end of study, 9.5 h after end of hypoxia. Two mL of blood were drawn each time, replaced by 2 mL of 0.9% NaCl. Samples were immediately spun for 10 min at 4°C and plasma was separated from the cells. These samples were snap frozen in liquid nitrogen before storage at –80°C. Samples were shipped on dry ice to Valencia, Spain for analysis. The samples were blinded prior to shipment. When measuring lipid peroxidation, we assessed F_2-isoprostanes (8-iso-15(R)-$PGF_{2\alpha}$, 1a, 1b-dihomo-$PGF_{2\alpha}$, 2, 3-dinor-$IsoPsF_{2\alpha}$-III, 8-iso-15-keto-PGE_2, 8-iso-15-keto-$PGF_{2\alpha}$, 8-iso-PGE_2, 5-$IsoPsF_{2\alpha}$-VI, 8-iso-$PGF_{2\alpha}$), isofuranes, F_4-neuroprostanes and neurofuranes. The samples were subject to UPLC-MS/MS [4], although the F_4-neuroprostanes and neurofuranes are methodologically challenging to measure in plasma [19].

The bladder was emptied at 210 min and immediately prior to euthanization, 10 mL of urine were drawn, immediately snap frozen in liquid nitrogen and then stored at –80°C before shipment to Valencia. The samples were blinded prior to shipment. Urine was analyzed for the same lipid peroxidation products as in plasma using UPLC-MS/MS [20].

Statistics

All statistics were done using IBM SPSS Statistics 23 and GraphPad Prism 6 (Prism 6.0, GraphPad Software Inc., San Diego, CA, USA). The physiological data were normally distributed and are expressed as mean ± SD. One-way ANOVA analysis was performed with LSD post-hoc test. We have two groups eligible for comparison; the normothermia group and the hypothermia group, hence the use of LSD *post-hoc* test.

The data generated for lipid peroxidation analysis were all non-normally distributed and the log-transformation was performed together with the Shapiro-Wilk's test for normality. Where the log-transformed data were normally distributed, parametric tests were used. For the non-parametric statistics, we applied the Kruskal-Wallis test and the Mann-Whitney *U* test for analysis and data which are expressed as median with inter quartile range (IQR). Data for the experiments are displayed as box and whiskers plots with maximum, 75th percentile, median, 25th percentile and minimum. Table data are expressed as mean with standard deviation. P values <0.05 were considered statistically significant with a 95% confidence interval.

Results

Physiology

The groups were comparable as there were no intergroup differences in body weight, post-natal age, hypoxia time or baseline physiological (heart rate, MABP) and biochemical measures (blood lactate, base excess and glucose) at the time of the first and second randomization (Table 1). The mean hypoxia time was close to similar

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

Measurements	Hypoxia	DHA	Hypothermia	Hypothermia + DHA	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
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
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
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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of 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 of study	197 (53)	208 (53)	144 (30)	170 (46)	168 (42)	**0.019**

Values as mean (SD). ANOVA p-values <0.05 in bold typing. Sham piglets in italic typing did not go through hypoxia.

in all the groups: 41–42 min. During hypoxia, there were no significant differences between the groups when measuring pO_2, pCO_2, pH and lactate at nadir. Significant differences were found between hypoxia groups and the sham group only, as the sham group was kept normoxic for the entire experiment.

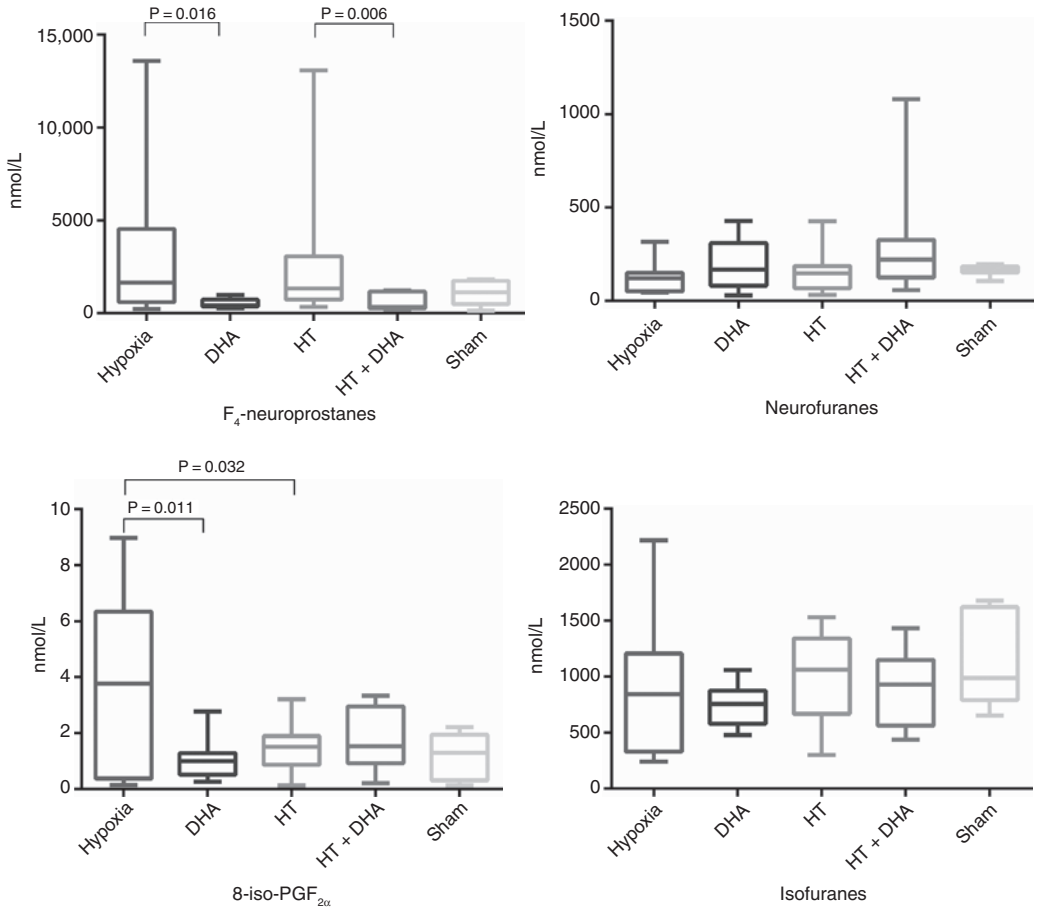

Figure 2: Peroxidation metabolites at end of study.
Box and whiskers plots showing measurements of F_4-neuroprostane, neurofurane, F_2-isoprostane and isofurane in urine at end of study.

Blood pressure

Globally, the ANOVA analysis did not show any significant differences (P = 0.10) between the groups at 210 min, but nominally, there was a significant difference between hypothermia vs. hypothermia + DHA, P = 0.041. From 210 min to end of study, the hypothermia group dropped from 61.9 (±10.4) to 49.9 (±9.7) mm Hg compared to the hypothermia + DHA which dropped from 53.4 (±10.5) to 51.6 (±13.0) mm Hg). This gave a significant fall in blood pressure in the hypothermia group compared to the hypothermia + DHA group (mean 10.2 ± 4.1 mm Hg, 95% CI: 1.8–18.6, P = 0.020). No statistically significant blood pressure changes were detected in the normothermia groups.

Urine samples

F_4-neuroprostanes, F_2-isoprostanes, neurofuranes and isofuranes were measured in urine at 210 min after end of hypoxia and at end of study.

There were no significant differences between any of the intervention groups at 210 min immediately prior to DHA administration.

At end of study, the F_4-neuroprostanes were significantly reduced (Kruskal-Wallis test, P = 0.006) in the groups receiving DHA compared to those not receiving DHA (Figure 2). F_4-neuroprostanes were significantly lower in the DHA group (median, IQR 440 nM, 367–738) compared to the hypoxia group (1652 nM, 610–4557, P = 0.016). For the hypothermia treated animals, the F_4-neuroprostanes were also reduced in the hypothermia + DHA group (median, IQR 356 nM, 264–1180) compared to the hypothermia group (median, IQR 1338 nM, 744–3085, P = 0.006). There was no significant difference in F_4-neuroprostanes when comparing hypoxia (median, IQR 1652 nM, 610–4557) to hypothermia (median, IQR 1338 nM, 744–3085, P = 0.91) (Figure 3).

The isoprostane compound 8-iso-$PGF_{2\alpha}$ was significantly reduced in the DHA group compared to the hypoxia group (mean difference 2.34 nM, 95% CI: 0.58–4.1, P = 0.011) (Table 2). There was no significant difference between the

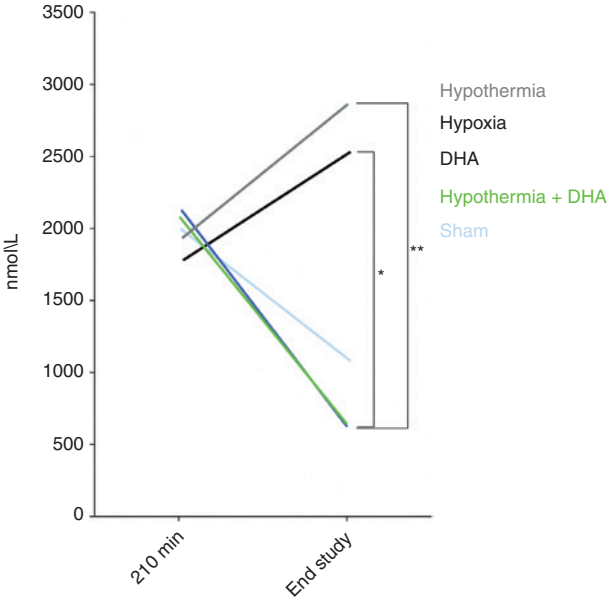

Figure 3: Time development of F_4-neuroprostanes.
Graph shows development of F_4-neuroprostanes from 210 min until end of study (mean values). Lipid peroxidation increases in non-DHA receiving intervention groups whereas it decreases in sham and DHA groups (P = 0.019 ANOVA, repeated measures). *Hypoxia vs. DHA, P = 0.020 and **Hypothermia vs. Hypothermia + DHA, P = 0.012 for mean difference in F_4-neuroprostanes from 210 min to end of study.

hypothermia groups. Overall F_2-isoprostanes including the seven other isoprostane compounds showed no significant differences between the groups at end of study. Also for isofuranes and neurofuranes, there were no significant differences between the groups at end of study.

Plasma samples

Sixty-nine percent of the F_4-neuroprostane samples and 56% of the neurofurane samples were under the level of detection (LOD) in plasma. We were therefore unable to perform a subgroup analysis of these metabolites.

The F_2-isoprostanes were by contrast, easily detectable at both timepoints. When we pooled the groups, we found that F_2-isoprostanes generally increased from 210 min (median, IQR 75,323 nmol, 42,268–147,771) to end of study (117,453 nmol, 55,144–204,425, P = 0.005). There were however, no significant differences in any of the isoprostane compounds or total F_2-isoprostanes between the intervention groups (ANOVA, P = 0.30).

Exclusions and mortality

We had to exclude two piglets from the study. One was due to massive cerebral edema and autolysis upon autopsy (as noted in the manuscript), the brain was not possible to extract as a whole. The piglet also had an anemic liver and pulmonary bleedings. It had shown signs of severe hemodynamic alterations (acidosis and hypotension) for several hours prior to euthanization. The other piglet was excluded just following hypoxia as by mistake, the piglet was resuscitated with 100% O_2 for 15 min before the error was detected.

We had a mortality of 8% in the study, all but one related to hypoxia where the piglets developed hypotension and arrhythmia and did not respond to resuscitation. One piglet was very difficult to intubate (probably subglottic stenosis) and the piglet died due to complications with intubation.

Discussion

In our neonatal asphyxia model, we found significantly reduced F_4-neuroprostanes in both normothermic and hypothermic piglets that were treated with DHA, indicating decreased lipid peroxidation. We also found a

Table 2: Lipid peroxidation in urine at end of study.

	Group									
	Hypoxia		DHA		Hypothermia		DHA+Hypothermia		Sham	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
F_4-neuroprostanes[a]	**2522**	2979	**624**	351	**2876**	3832	**623**	447	1087	698
F_2-isoprostanes	1281	1271	1554	1001	1542	1055	1425	1333	2325	2571
8-iso-PGF2[b]	**3.44**	3.37	**1.09**	0.77	**1.50**	0.91	1.78	1.11	1.16	0.85
Neurofuranes	128	88	194	134	158	110	314	298	163	29
Isofuranes	902	619	773	209	995	389	899	326	1150	418

Bold values refer to the statistically significant comparisons. [a]DHA is significantly reduced compared to Hypoxia. DHA + Hypothermia is significantly reduced compared to Hypothermia, [b]Both DHA and Hypothermia are significantly reduced compared to control.

significantly reduced level of the most abundant isomer 8-iso-$PGF_{2\alpha}$ in the DHA treated group.

The combination of hypothermia and DHA has to our knowledge previously only been described in rodents. Berman et al. [17] have shown that post-HI treatment with therapeutic hypothermia and DHA reduced brain damage in rats. That finding is supported by results from this study in terms of reduced oxidative stress in piglets receiving DHA.

Lipid peroxidation

Prior to DHA administration, there was no difference between the groups when measuring urine F_4-neuroprostane ($P = 0.24$). The bladder was emptied at 210 min through a bladder tap, so the results found at end of study suggest effect of DHA. We could not confirm a reduced level of F_4-neuroprostane in plasma at end of study because the majority of samples were under the level of detection.

F_4-neuroprostanes are considered to be a marker of brain oxidative stress as DHA is largely found in the cerebral cortex and retina [21]. Solberg et al. [22] found significantly reduced F_2-isoprostanes and F_4-neuroprostanes in the prefrontal cortex of HI piglets treated with DHA. DHA is, however, also present in tissues outside the central nervous system (CNS) like red blood cells, spleen and liver, so we cannot exclude the possibility of our results reflecting reduced lipid peroxidation in other organs as well. It has been questioned whether supplementary PUFA can actually increase lipid peroxidation. In a clinical RCT by Shichiri et al. [23], they found reduced lipid peroxidation in the plasma and red blood cells of hypertricgyceridemic men when the concentration of ingested DHA increased. On the other hand, Dupuy et al. [24] found that supplementation of DHA to atherosclerotic mice gave increased levels of F_4-neuroprostanes in brain tissue, though this was in part thought to be due to the replacement of arachidonic acid by DHA in the membrane phospholipids.

8-iso-$PGF_{2\alpha}$, a member of the F_2-isoprostane family, has been well-accepted as a valuable biomarker for the assessment of oxidative stress [25]. Ahola et al. [26] found elevated levels of 8-epi-$PGF_{2\alpha}$ in plasma samples of extremely low birth weight infants who later developed periventricular leukomalasia. We found significantly reduced levels of 8-iso-$PGF_{2\alpha}$ in urine in both the DHA and the hypothermia group compared to the hypoxia group. This indicates DHA and hypothermia having a significant effect on reducing oxidative stress following HI. Other isomers have also been suggested as biomarkers of perinatal asphyxia. In a recent study by Chafer-Pericas et al. [7], they suggest the potential of 8-iso-15(R)-$PGF_{2\alpha}$ as a biomarker of neonatal encephalopathy. This is based on them finding a significant increase in total F_2-isoprostanes and this isomer (but no increase for the 8-iso-$PGF_{2\alpha}$ compound) in the cord blood serum of acidemic infants. We did not find reduced F_2-isoprostanes in plasma following DHA administration in our study.

Both neurofuranes and isofuranes contain a tetrahydrofurane ring and they are generated by a reaction with molecular oxygen. Solberg et al. [27] found significantly increased levels of both neurofuranes and isofuranes when newborn piglets were resuscitated with 40% and 100% O_2. In our set-up, all intervention groups were resuscitated without supplementary oxygen to minimize the oxidative stress after HI. This may be the reason for no significant differences in the iso- and neurofurane groups.

Hypothermia

When assessing the effect of hypothermia on lipid peroxidation, we found only significance between the hypoxia group and the hypothermia group when measuring 8-iso-$PGF_{2\alpha}$. Significant findings could not be reproduced between the hypothermia group and the hypoxia group for any of the other peroxidation products. The effect of hypothermia on lipid peroxidation is not fully established in the literature and we could not find any meta-analysis on the subject. Some studies [28–30] have shown that hypothermia in fact increases lipid peroxidation, one of the mechanisms being that the hemoglobin dissociation curve shifts to the left during hypothermia giving less oxygen to the tissues and increasing ROS production. Another aspect is that the cerebral circulation is lowered in response to the reduced metabolic demands in the hypothermic brain and this can both induce and reduce ROS production. On the other hand, a study by Bayir et al. [31] showed a marked gender effect of hypothermia on lipid peroxidation following traumatic brain injury where cooled male subjects had reduced levels of F_2-Isoprostanes in CSF, but females did not. We could not find any gender differences in our study. Then again Maier et al. [32] showed reduced ROS and markers of oxidative stress in rats during hypothermia. The present study finds that hypothermia reduces 8-iso-$PGF_{2\alpha}$ in urine, but for the other compounds measured, hypothermia neither decreases nor increases lipid peroxidation.

Physiology

Non-significant physiological differences between the groups indicate that they were statistically comparable

when they were randomized to different interventions and strengthen our findings. The hypothermia groups had, however, significantly different blood pressure levels at 210 min but not at end of study, suggesting that DHA had no impact on blood pressure. Hypothermia is known to both increase and decrease blood pressure [33, 34] and the significant difference between the hypothermia groups in this study is most likely random.

Limitations

There is a short follow-up time in our model as the piglets were euthanized 9.5 h after end of hypoxia. This limits the possibility to evaluate neuroprotective effects in the brain as these take time to develop. The operators were not blinded to the intervention groups during the experiment itself, but all samples were blinded prior to analysis.

Conclusion

DHA significantly reduces oxidative stress following HI by measures of F_4 –neuroprostanes in both normothermic and hypothermic piglets. Hypothermia reduces 8-iso-$PGF_{2\alpha}$, but none of the other lipid peroxidation compounds are affected by the hypothermia treatment. To assess whether DHA may have neuroprotective properties and be a tool in the search for additional treatment of HIE, more studies are required.

Author statement

Conflict of interest: Authors state no conflict of interest.

Material and Methods: Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human subject use has complied with all the relevant national regulations, and institutional policies, and is in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

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