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Oxidised fish oil in rat pregnancy causes high newborn mortality and increases maternal insulin resistance

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Short title: Adverse effects of oxidised fish oil in rat pregnancy

Abbreviation list: 8-OH-2dG, oxidation by-product 8-hydroxy-2-deoxy guanosine; AST, aspartate aminotransferase; CK, creatinine kinase; CV, coefficients of variation; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acids; LDH, lactate dehydrogenase; n-3 PUFAs, omega-3 polyunsaturated fatty acids; OxFO, group treated with oxidised fish oil; SOD, superoxide dismutase; UnFO, group treated with unoxidised fish oil.

ABSTRACT

Fish oil is commonly taken by pregnant women, and supplements sold at retail are often oxidised. We aimed to assess the effects of supplementation with oxidised fish oil during pregnancy in mothers and offspring, using a rat model, focussing on newborn viability and maternal insulin sensitivity. Female rats were allocated to a control or high-fat diet and then mated. They were subsequently randomised to receive a daily treatment of 1 ml of unoxidised fish oil, a highly oxidised fish oil, or control (water) throughout pregnancy by gavage. At birth, the gavage treatment was stopped, but the same maternal diets were fed *ad libitum* throughout lactation. Supplementation with oxidised fish oil during pregnancy had a marked adverse effect on newborn survival at day 2, leading to much greater odds of mortality than in the control (odds ratio 8.26) and unoxidised fish oil (odds ratio 13.70) groups. In addition, maternal intake of oxidised fish oil during pregnancy led to increased insulin resistance at the time of weaning (three weeks after exposure) compared to control dams (HOMA-IR 2.64 vs 1.42; p=0.044). These data show that the consumption of oxidised fish oil is harmful in rat pregnancy, with deleterious effects in both mothers and offspring.

Keywords: omega-3; n-3 PUFA; lipid peroxides; lipids; polyunsaturated fatty acids; dams; pups

INTRODUCTION

Fish oils are rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs) and are the most common non-vitamin non-mineral supplements in the United States, taken by 10% of adults (7). They are frequently consumed during pregnancy because of claimed benefits to neurodevelopment of the offspring, particularly in childhood. However, the evidence for neurodevelopmental benefits from n-3 PUFAs intake during pregnancy is poor (42), and a recent report showed no effects on objective assessments of cognition, language, and executive function at 4 years of age (44). Furthermore, a recent systematic review and meta-analysis showed no benefit of n-3 PUFAs during pregnancy on pre-eclampsia, intra-uterine growth retardation, post-partum depression, or childhood development (53). In contrast, a number of animal studies have shown adverse effects on neurological development of the offspring following a maternal diet rich in n-3 PUFAs (13, 14, 30, 51), although in some the dose would be unrealistic for a human diet (13, 14). Nonetheless, in New Zealand for example, 15% of women undergoing fertility treatment (27) and approximately 20% of pregnant women (20) were taking fish oil. The global consumption of n-3 PUFA supplements continues to grow, despite the increasing number of studies failing to show improvements in hard outcomes (28).

Pregnancy is a critical time period when considering the safety of medications, and the same should apply to dietary supplements. Fetal life is a highly vulnerable stage, when complex embryological

processes can be disrupted by external factors (such as chemicals) that may be harmless to the mother (62). Further, perinatal factors play an important role in programming body composition and cardio-metabolic risk later in life (26, 32).

The n-3 PUFAs in fish and other marine oils (e.g. krill, calamari, and algae) are easily oxidised to a variety of lipid peroxides and other secondary oxidation products (54). In a recent comprehensive survey of fish oil products on the New Zealand market, 83% exceeded the recommended level of lipid peroxides, 33% by more than two fold (4). In addition, 25% exceeded the recommended level of secondary oxidation products (aldehydes and ketones) (4). Similar results have been demonstrated in studies from North America (36, 50) and Africa (48). This implies that most women taking fish oil during pregnancy are routinely exposed to oxidation products. Further, antioxidant status is reduced during pregnancy (24) and extremely low in the early embryo (60), so that pregnancy may be a time of special vulnerability to lipid peroxides. However, there is insufficient evidence to establish a safe level of oxidation in fish oils for consumption (2, 19).

In animal models, exposure to oxidised lipids has been shown to cause harm, including growth retardation, organ toxicity, and accelerated atherosclerosis (3, 22, 57). However, the effects of consuming oxidised lipids during pregnancy are unknown (2). For this reason, in a study primarily designed to investigate the effects of fish oil supplementation during pregnancy on the adult offspring of rats fed a high-fat diet, we have also included oxidised fish oil groups. As obesity is associated with increased oxidative stress (23) and greater production of oxidised lipids (37), the effects of an oxidised lipid supplement may be more marked in obese mothers. This manuscript describes the effects of the oxidised fish oil on neonatal viability and maternal insulin sensitivity, while examining for a possible interaction with a maternal obesogenic diet.

METHODS

Ethics

Ethics approval was granted by the Animal Ethics Committee at the University of Auckland (Approval 001175). This study was performed in accordance with all appropriate institutional and international guidelines and regulations for animal research.

Study design

Female Sprague-Dawley rats (n=75) were housed under standard conditions at 25^{o}C with a 12-h light: 12-h dark cycle. Animals were assigned to one of two isocaloric diets *ad libitum* for ten days prior to

mating (Figure 1): high-fat diet (D12451, Research Diets Inc, New Brunswick, NJ, USA) containing 45% kcal as fat, 35% as carbohydrate, and 20% as protein; or control diet (D12450H) containing 10% kcal as fat, 70% as carbohydrate, and 20% as protein. The two diets had the same amount of sucrose (17% of kcal) and were otherwise nutrient-matched. Neither diet contained the long-chain n-3 PUFAs eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Note that the maternal high-fat diet adopted approximated commonly consumed western style diets and has metabolic effects similar to maternal obesity (33).

Female rats were time-mated using an oestrous cycle monitor (EC-40, Fine Science Tools, San Francisco, CA, USA) at a mean age of 113 days. Day 1 of pregnancy was determined by detection of spermatozoa by vaginal lavage, when pregnant dams were individually housed, continued on the study diet, and were allocated to one of three treatment (gavage) groups: unoxidised fish oil (UnFO), oxidised fish oil (OxFO), or water (Control). On each day of pregnancy, 1 ml of the treatment was administered by oral gavage (Figure 1). Control rats underwent water gavage to ensure that dams in all groups were exposed to the same experimental manipulation. Note that an oil was not used as the control treatment in this study, as saturated (38), monounsaturated (18, 39), and polyunsaturated (18, 39) fatty acids all have potentially important metabolic effects, so that any oil used as a control could potentially have had effects on maternal metabolism or offspring phenotype. Further, water gavage is a standard control treatment in studies of n-3 PUFA rich oils in rodents (5, 12, 43, 45).

Food intake and body weight were recorded every 3rd day. At the time of birth, the gavage treatment was stopped, but the same maternal diets were fed *ad libitum* throughout lactation (until postnatal day 21) (Figure 1). On postnatal day 2, offspring were counted, sexed (by measuring anogenital distance), weighed and measured; all dead pups were sexed and counted; litter size was randomly adjusted to 8 pups to ensure standardized metabolic demand on individual dams. Pups not allocated to litters were killed by decapitation, and plasma, packed blood cells, liver, and heart were collected for analyses (Figure 1).

Offspring were weaned on day 21 (Figure 1). The day after weaning, dams were fasted overnight and killed by sodium pentobarbitone anaesthesia (60 mg/kg; intraperitoneal) followed by decapitation. Blood was collected in heparinised tubes, and stored on ice until centrifugation and removal of plasma for analysis. Liver, heart, and the retroperitoneal fat pads were collected and weighed. Fasting glucose and insulin were used to calculate HOMA-IR (10), which is a validated surrogate measure of insulin sensitivity in rats that is well-correlated (r=0.71) with the hyperinsulinaemic euglycaemic clamp (10).

Treatment oil

The treatment fish oil was produced from hoki (*Macruronus novaezelandiae*) liver (Seadragon Marine Oils, Nelson, New Zealand). Half of the oil was frozen and used as the 'unoxidised fish oil' (UnFO). The remainder was treated by bubbling oxygen for 30 days under a fluorescent lamp at room temperature to produce the 'oxidised fish oil' (OxFO). Oxidation was assessed by measuring the peroxide value (PV) and anisidine values (AV) (15). The unoxidised oil had a PV of 1.5 meq/kg and AV of 0.6, which are well within recommended indices of oxidation (25, 31, 58) and lower than those of any fish oil available on the New Zealand market (4). In contrast, the oxidised oil had PV of 48.8 meq/kg and AV of 4.5, greatly exceeding recommended levels (25, 31, 58). The fatty acid content of both oils was measured independently by gas chromatography (Table 1). The UnFO and OxFO had similar concentrations of fatty acids, except for a small reduction (10%) in EPA and DHA concentrations in the OxFO oil. Thus, the treatment oils differed primarily in the concentration of lipid peroxides. The study oils were stored in small aliquots, frozen, sealed, and stored in darkness. Each day a new aliquot was thawed prior to use, so that further oxidation of oils prior to gavage was minimised. This is important as if fish oil is mixed into food, the peroxide value increases significantly over 24 hours in the food bowl (47).

Assays

Creatinine kinase (CK), aspartate aminotransferase (AST), free fatty acids (FFA) and lactate dehydrogenase (LDH) were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) with all coefficients of variation (CV) less than 6%. Insulin was measured by rat-specific ELISA (Crystal Chem, Illinois, USA) with CV of 11%. Total superoxide dismutase (SOD) activity was measured with a commercial enzyme assay kit as per the manufacturer's instructions (Sapphire Bioscience, Mulgrave, Australia) with a CV less than 6%. An EIA kit was used to measure the DNA oxidation by-product 8-hydroxy-2-deoxy guanosine (8-OH-2dG) as a marker of oxidative stress (59). DNA was extracted from samples using a DNA isolation kit (Promega, Sydney, Australia). Each sample was then diluted, so that 50 mg of DNA were used in the 8-OH-2dG assay, which was carried out as per the manufacturer's instructions (Sapphire Bioscience, Mulgrave, Australia) with a CV of 3.5%.

Statistical analyses

Rat dam outcomes were compared between groups using general linear regression models. Newborn survival was assessed using generalized linear mixed models, including the dam identification code as a random factor. Offspring outcomes were assessed using random effects mixed models, also including the dam identification code as a random factor. Dietary intake over the course of the study was assessed using linear regression models with repeated measures. Note that all models assessing the effects of

gavage also adjusted for diet (high-fat or control). The associations between SOD and 8-OH-2dG were evaluated using Pearson's correlation coefficients and simple linear regressions. Statistical analyses were carried out in SAS v.9.4 (SAS Institute, Cary, NC, USA) and Minitab v.16 (Pennsylvania State University, State College, PA, USA). All tests were two-tailed with significance level maintained at 5%. Unless stated otherwise, data in the text are means.

RESULTS

There was no evidence of an interaction between gavage treatment and maternal diet for the two main outcomes of interest (maternal insulin sensitivity and newborn survival) or most other study parameters. Thus, to better characterise the effects of oxidised fish oil and to increase statistical power, the dams and litters exposed to the same gavage treatment were pooled into the three groups: UnFO, OxFO, and Control.

Pregnancy & newborn survival

Of the 75 rat dams that were mated, 49 became pregnant and successfully had pups, including 17 dams in Control, 17 in UnFO, and 15 in OxFO. Supplementation (fresh fish oil, oxidised fish oil, or water) had no effect on litter numbers, sex ratio of the offspring, or frequency of non-pregnancy. However, oxidised fish oil during pregnancy had a marked adverse effect on offspring survival at day 2, leading to much greater odds of mortality than in the Control [odds ratio 8.26 (95% CI 2.04–33.33)] and UnFO [odds ratio 13.70 (95% CI 3.08–62.50)] groups. Notably, the effects of oxidised fish oil on neonatal mortality were nearly identical irrespective of maternal diet (Figure 2).

Offspring outcomes

Amongst pups who were alive at day 2, supplementation had no effect on body weight of live pups, but UnFO pups were longer than controls (+1.3 mm; p=0.041) (Table 2). There were no differences in relative organ weights amongst the culled offspring at day 2, or in random blood glucose, FFA, total protein, AST, CK or LDH concentrations (Table 3). However, SOD activity was higher in the UnFO group (0.20 U/ml) compared with Control (0.14 U/ml; p=0.001) and OxFO (0.15; p=0.005) pups, while 8-OH-2dG levels were lower in the UnFO group (48 pg/ml) compared with Control (63 pg/ml; p=0.023) (Table 3). In addition, SOD activity and 8-OH-2dG were inversely correlated among UnFO pups (r=-0.65; p=0.001), but not among the other two groups (Figure 4).

Maternal outcomes

At weaning (postnatal day 21), dams in the three gavage groups were of similar size and had similar organ weights (data not shown). However, dams supplemented with oxidised fish oil had higher fasting blood glucose concentrations (5.56 mmol/l) than dams from Control (4.80 mmol/l; p=0.012) and UnFO (4.72 mmol/l; p=0.007) groups (Table 2). Dams from the oxidised group were also more insulin resistant than controls, as indicated by differences in HOMA-IR (p=0.044; Table 2).

Maternal high-fat diet vs control diet

There was no evidence of an interaction between maternal diet and gavage treatment for the main study outcomes, pregnancy parameters, litter characteristics, or most other outcomes examined. A significant interaction between diet and gavage was only observed for SOD activity and liver weight (Table 4). Unoxidised fish oil gavage led to increased SOD activity ($p<0.001$ vs Control; $p<0.0001$ vs OxFO), but only amongst pups exposed to a maternal control diet (Table 4). Unoxidised fish oil led to a reduction in relative liver weight of pups only when the dam consumed a high-fat diet (p=0.022 vs Control; p=0.020 vs OxFO). This was not observed with the control diet (Table 4). Among rat dams fed a control diet, OxFO dams had higher fasting glucose (p=0.007) and tended to be more insulin resistant (p=0.051) than those who received control gavage (Table 4). There appears to have been a similar effect amongst dams fed a high-fat diet, but there was reduced statistical power due to lower numbers of dams in the OxFO group (Table 4).

DISCUSSION

In this trial of fish oil supplementation during rat pregnancy, oxidised fish oil led to an 8- to 13-fold increase in the odds of newborn mortality. Importantly, there was no increase in mortality in the groups fed unoxidised fish oil compared to controls, indicating that the adverse effect on newborn survival was due to the presence of lipid oxidation products. Further, dams exposed to the oxidised fish oil in pregnancy were more insulin resistant at weaning, which was the end of the study and 21 days after last exposure. However, the effects of the oxidised fish oil on the newborn pup survival did not differ between dams consuming a high-fat or a control diet. This suggests that the known adverse effects of a high-fat diet on maternal metabolism (17, 33) did not alter the toxicity of oxidised fish oil to newborn pups. These data show that lipid peroxides formed in oil rich with n-3 PUFA are harmful in rat pregnancy, raising the possibility that they may also have an adverse effect in human pregnancy.

The oxidised fish oil was characterised by a high concentration of lipid peroxides, which could have caused oxidative stress (and the consequent toxic effects). Oxidative stress has important effects during pregnancy, as it contributes to the teratogenic effects of drugs (61) and has a role in complications such as spontaneous abortion, pre-eclampsia and intrauterine growth restriction (1). Oxidised lipids influence placental development, lipid metabolism, and lipid transport (37), and lipid peroxide levels are markedly higher in serum and placenta of pre-eclamptic pregnancies (24). Thus, in principle, the increased newborn mortality in the OxFO group could have been mediated by effects on maternal and placental health. Alternatively, lipid peroxides could have crossed the placenta and directly affected the fetus, but there is only weak evidence to suggest that lipid peroxides can cross the placenta (52).

Our findings indicate that the redox effects of oxidised and unoxidised fish oils on the pups differed. However, it was not possible to determine the mechanisms underlying the increased newborn mortality, or whether oxidative stress played a role. Nonetheless, previous studies showed that fish oil supplementation increases endogenous antioxidant enzymes (21) and reduces oxidative damage (16, 21). Consistent with these studies, supplementation with unoxidised fish oil was associated with reduced 8-OH-2-dG levels (a marker of oxidative DNA damage in the pups) and increased activity of superoxide dismutase (SOD, a key antioxidant enzyme). Further, 8-OH-2-dG and SOD activity were negatively correlated among the UnFO pups. In contrast, neither these beneficial effects nor the correlation between 8-OH-2-dG and SOD were observed in the OxFO pups. It should be noted that due to limited amount of blood samples that can be extracted from newborn pups, markers of lipid and protein oxidative damage could not be measured.

Surprisingly, and at odds with the high newborn mortality in the OxFO group, no differences were identified between groups for a range of markers of cellular toxicity (e.g. FFA, CK, and LDH) or in body weight or relative organ mass. However, the earliest samples collected were from offspring alive on postnatal day 2 (at the time of a planned cull to standardize litter size), when a large number of pups had already died. Consequently, there was a 'healthy survivor' bias, i.e. the most severely affected pups had died and could not be assessed, so that we sampled those least affected by the toxicity of the oxidised fish oil.

Interestingly, oxidised fish oil supplementation dams led to greater insulin resistance in dams 3 weeks beyond the last dose. Furthermore, as the mothers were culled at this stage, we do not know whether this effect would have been sustained even longer (i.e. a long-term reprogramming of metabolism). Conversely, no insulin-sensitising effect of unoxidised fish oil was apparent 3 weeks after the end of supplementation. Short-term improvements in insulin sensitivity with n-3 PUFAs have been demonstrated in rodents (34, 46, 56), but it is likely that this effect was lost in our unoxidised fish oil group over time. Nonetheless, we report for the first time that oxidised fish oil increases insulin

resistance, the opposite effect reported for unoxidised fish oil (34, 46, 56). The cause of this increased insulin resistance is unknown, but it could be due to induction of systemic inflammation, as oxidative stress activates the proinflammatory NF-κB pathway (41), which is central to the inflammation associated with obesity and insulin resistance (8, 35, 55).

Our findings cannot be extrapolated directly to humans. However, it is clear that the dose of lipid peroxides used in this study was high. For a woman to consume an equivalent dose of lipid peroxides [i.e. 890 meq/m^2/day based on surface area (49)] from a retail supplement (4), she would have to take at least 40 ml of oxidised fish oil per day. Thus, it is very unlikely that women taking fish oil during pregnancy would be exposed to such a high dose of lipid peroxides. Nevertheless, no safe level of peroxide exposure has been established in humans, and pregnancy is a time of special vulnerability, where endogenous antioxidant status is reduced (24).

Many possible causes for the variability of outcomes in trials of n-3 PUFAs in pregnancy have been raised (11). However, the potential for oxidation to change the biological activity of trial oils has not been recognised (2). As oxidised fish oil induced maternal insulin resistance in this study, it is possible that trials and meta-analyses assessing the effects of fish oil on a range of outcomes (including gestational diabetes, birth weight, and offspring metabolism) may have been confounded by oxidation (2).

Our study had a number of strengths. First, we used an oxidised fish oil that was derived from the fresh fish oil, and thus identical except for the presence of oxidation products. Second, we utilized small frozen aliquots of trial oils with delivery by gavage, which ensured the correct dose was given, and that inadvertent oxidation of the unoxidised oil did not occur. Third, the control dams also underwent daily gavage to prevent confounding by the stress of the procedure itself (9). However, this study also has limitations. It was not designed to identify the mechanism of harm, and assessment of pups on day 2 (after 25% of pups had died in the OxFO group) introduced a 'healthy survivor' bias, and could not discriminate between stillborn pups and those that died after delivery. Further, the dose used and the severity of oxidation was relatively high, and the possible effects of lower doses in human pregnancy cannot be extrapolated.

Perspectives and significance

This is the first study to show negative effects of oxidised fish oil in pregnancy, specifically, increased maternal insulin resistance and increased newborn mortality in rats. It represents an important proof of concept that supplementation with oxidised fish oil in pregnancy may be harmful. This is relevant as fish oil supplements are often oxidised at the time of purchase (4, 36, 48, 50), and are commonly taken

during pregnancy. Therefore, there is a pressing need for studies examining dose-response relationships between oxidised fish oil consumption and pregnancy outcomes. Given our findings, the lack of compelling evidence that fish oil supplementation is beneficial in human pregnancy (53), and previous studies showing adverse effects (6, 29, 40, 44), caution is advised when considering taking fish oil supplements in pregnancy. However, it should be pointed out that our findings are unlikely to apply to fresh oily fish. Clinical trials of n-3 PUFAs should ensure that adopted supplements are independently verified to be unoxidised, and their oxidative state should be reported. Lastly, future studies should focus on three particular aspects: 1) whether there is a dose-response relationship between lipid peroxide intake and both maternal and newborn outcomes, and whether doses more comparable to human supplementation are also harmful; 2) whether the addition of antioxidants can mitigate any adverse outcomes; and 3) the identification of the exact mechanisms leading to newborn mortality (including a wider range of markers of oxidative stress and assessments in fetal life).

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Figure 1. Diagram describing individual steps of the trial and indicating the timing of assessments. T1: Virgin female rats were randomised to control or high-fat diet. T2: Females were mated and further randomised to one of the three gavage treatments, i.e. unoxidised fish oil (UnFO), oxidised fish oil (OxFO), or water (Control). T3: on postnatal day 2 pups were counted, sexed and weighed; excess pups were culled and samples taken. T4: on postnatal day 21 pups were separated, while dams were culled and had samples taken. Values in boxes represent the number of dams, except at T3 where the total number of pups at day 2 is also provided in parentheses.

Figure 2. Overall mortality at postnatal day 2 among rat pups born to dams fed a control or high-fat diet, which were supplemented during pregnancy via gavage with water (Control), unoxidised fish oil (UnFO), or oxidised fish oil (OxFO).

Figure 3. The association between superoxide dismutase (SOD) activity and 8-OH-2dG concentrations in the packed blood cells of rat pups exposed to maternal gavage with water (A), unoxidised fish oil (B), or oxidised fish oil (C). Blood samples were collected from rat pups that were alive at day 2 and culled on this day.

Table 1. Fatty acid concentrations in fresh fish oil (UnFO) and oxidised fish oil (OxFO) supplements determined by gas chromatography–mass spectrometry (from analysis of 6 replicates), and their respective oxidative indices (from 3 replicates). Data are means ± standard errors, from analysis of 6 replicates.

	UnFO	OxFO	p-value
Fatty acid concentration (mg/g of oil)			
C16:0 (palmitic acid)	105.53 ± 2.01	100.66 ± 4.08	0.87
C16:1n-7 (palmitoleic acid)	15.87 ± 0.34	14.96 ± 0.62	0.20
C18:0 (stearic acid)	28.26 ± 0.56	26.47 ± 1.39	0.34
C18:1n-7 (cis-vaccenic acid)	4.35 ± 0.05	4.10 ± 0.16	0.055
C18:1n-9 (oleic acid)	425.59 ± 7.99	400.97 ± 15.3	0.42
C18:2n-6 (linoleic acid)	3.12 ± 0.04	3.04 ± 0.08	0.42
C18:3n-3 (α-linolenic acid)	5.52 ± 0.05	5.32 ± 0.32	0.26
C18:3n-6 (γ-linolenic acid)	1.68 ± 0.24	1.54 ± 0.19	0.52
C20:0 (arachidic acid)	0.72 ± 0.01	0.68 ± 0.03	0.26
C20:1n-9 (eicosenoic acid)	0.47 ± 0.01	0.43 ± 0.03	0.15
C20:2n-6 (eicosadienoic acid)	1.67 ± 0.03	1.53 ± 0.07	0.11
C20:3n-6 (dihomo-γ-linolenic acid)	4.10 ± 0.11	3.79 ± 0.23	0.42
C20:4n-6 (arachidonic acid)	2.90 ± 0.08	2.80 ± 0.18	0.75
C20:5n-3 (eicosapentaenoic acid; EPA)	42.53 ± 0.76	38.34 ± 1.58	0.004
C22:5n-3 (docosapentaenoic acid)	1.09 ± 0.03	1.21 ± 0.18	0.87
C22:6n-3 (docosahexaenoic acid, DHA)	89.00 ± 1.77	79.87 ± 3.35	0.006
Oxidative Indices			
Peroxide value (meq/kg)	1.46 ± 0.03	48.81 ± 1.63	<0.0001
Anisidine value	0.62 ± 0.03	4.53 ± 0.43	0.006

Table 2. Outcomes among rat dams and their respective litters according to type of supplementation during pregnancy: water (Control), unoxidised fresh fish oil (UnFO), and oxidised fish oil (OxFO). Daily energy intake includes the caloric value of the gavage treatment (i.e. oil or water). Data are means ± standard errors (except for data on glucose homeostasis that are geometric means and 95% confidence intervals), adjusted for diet (high-fat or control). *p<0.05 and **p<0.01 vs OxFO; †p<0.05 vs Control. Note that there was no observed interaction between maternal diet and gavage treatment for any of the listed parameters.

	Control	**UnFO**	**OxFO**
n	17	17	15
Weight at mating (g)	315 ± 8	319 ± 8	329 ± 8
Pregnancy			
Weight at day 22 (g)	446 ± 11	445 ± 11	455 ± 12
Pregnancy weight gain (%)	41.3 ± 2.5	39.9 ± 2.5	39.1 ± 2.7
Daily energy intake (kcal/g of body weight)	0.21 ± 0.01	0.23 ± 0.01	0.22 ± 0.01
Litter characteristics (day 2)			
Litter size (n)	12.8 ± 0.6	13.0 ± 0.6	12.4 ± 0.7
Sex ratio (% male per litter)	51 ± 3	46 ± 3	51 ± 3
Weight of live pups (g)	5.5 ± 0.2	5.9 ± 0.2	5.4 ± 0.2
Length of live pups (mm)	47.1 ± 0.4	48.4 ± 0.4†	47.4 ± 0.5
Dams post-weaning (day 22)			
Weight (g)	352 ± 7	343 ± 8	339 ± 9
Retroperitoneal fat weight (% body weight)	1.4 ± 0.1	1.2 ± 0.1	1.5 ± 0.1
Liver weight (% body weight)	4.7 ± 0.3	5.0 ± 0.3	4.3 ± 0.3
Fasting glucose (mmol/l)	4.80 (4.46–5.16)*	4.72 (4.38–5.09)**	5.56 (5.10–6.07)
Fasting insulin (pmol/l)	239 (167–343)	270 (186–393)	378 (240–595)
HOMA-IR	1.42 (0.98–2.07)*	1.58 (1.07–2.33)	2.64 (1.65–4.25)

Table 3. Characteristics of culled rat offspring on postnatal day 2 according to type of gavage supplementation during pregnancy: water (Control), fresh fish oil (UnFO), and oxidised fish oil (OxFO). Data are means ± standard errors, adjusted for diet (high-fat or control). Note that aside from glucose, all biochemical markers were performed in a reduced group, where pups were randomly selected from each litter. Also note that 4 pups in the OxFO group had blood taken for biochemistry but did not have organs weighed. **p<0.01 vs OxFO; †p<0.05 and ††p<0.01 vs Control. Note that an interaction between maternal diet and gavage treatment was only observed for liver weight and SOD.

		Control	UnFO	OxFO
n		46	36	20
Biochemical marker	Random blood glucose (mmol/l)	6.65 ± 0.65	6.22 ± 0.74	6.18 ± 0.93
Organ sizes	Brain (% body weight)	4.33 ± 0.12	4.50 ± 0.13	4.43 ± 0.16
	Heart (% body weight)	0.65 ± 0.03	0.71 ± 0.03	0.62 ± 0.04
	Liver weight (% body weight)	4.57 ± 0.15	4.51 ± 0.17	4.61 ± 0.21
n		24	24	24
Redox markers	SOD (U/ml)	0.14 ± 0.01	0.20 ± 0.01††**	0.15 ± 0.01
	8-OH-2dG (pg/ml)	63 ± 4	48 ± 5†	57 ± 5
Biochemical markers	Free fatty acids (mmol/l)	0.37 ± 0.04	0.29 ± 0.04	0.36 ± 0.05
	Total protein (g/dl)	2.26 ± 0.14	2.19 ± 0.14	2.38 ± 0.16
	AST (U/l)	397 ± 51	376 ± 51	340 ± 63
	CK (U/l)	114 ± 19	106 ± 18	119 ± 22
	LDH (U/l)	1767 ± 242	1418 ± 243	1461 ± 298

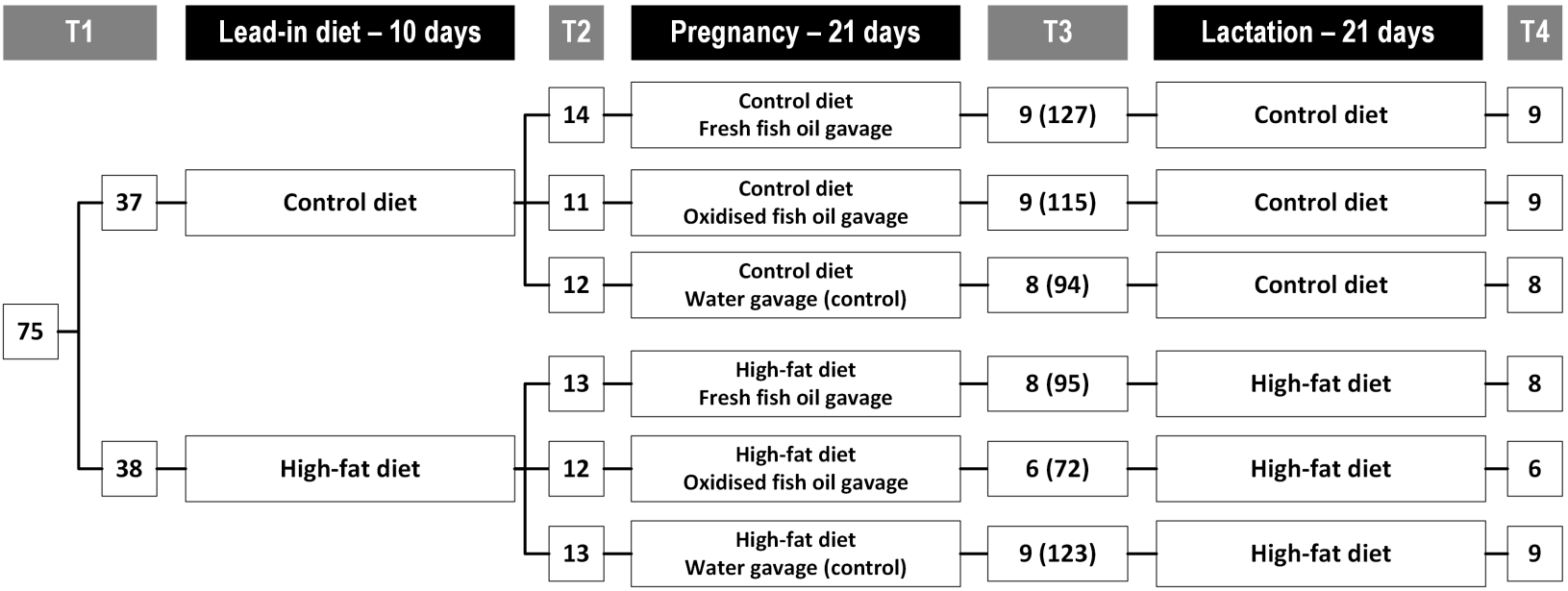
8-OH-2dG, DNA oxidation by-product 8-hydroxy-2-deoxy guanosine; AST, aspartate aminotransferase; CK, creatinine kinase; LDH, lactate dehydrogenase; and SOD, total superoxide dismutase.

Table 4. Study outcomes on rat dams (post-weaning day 22) and on culled rat offspring (postnatal day 2) according to maternal diet (Control or High-fat) and type of gavage treatment during pregnancy: water (Control), unoxidised fish oil (UnFO), or oxidised fish oil (OxFO). Data are means ± standard errors (except for data on glucose homeostasis that are geometric means and 95% confidence intervals), with offspring data adjusted for sex. Note that aside from glucose, all offspring biochemical markers were performed in a reduced group, where pups were randomly selected from each litter. Also note that two pups in each OxFO group had blood taken for biochemistry but did not have organs weighed.

		Control diet			High-fat diet		
		Control	**UnFO**	**OxFO**	**Control**	**UnFO**	**OxFO**
DAMS	n	8	9	9	9	8	6
	Weight (g)	332 ± 11	344 ± 11	334 ± 11	367 ± 9	344 ± 10	342 ± 13
	Liver weight (% body weight)	5.8 ± 0.5	6.2 ± 0.5	4.8 ± 0.5	3.6 ± 0.2	3.9 ± 0.2	3.8 ± 0.3
	Fasting glucose (mmol/l)	4.43 (4.01–4.89)	4.54 (4.12–5.02)*	5.44 (4.93–6.01)††	5.14 (4.60–5.73)	4.91 (4.36–5.52)	5.66 (4.84–6.61)
	Fasting insulin (pmol/l)	177 (101–311)	249 (142–438)	324 (172–608)	309 (184–519)	302 (174–525)	442 (212–918)
	HOMA-IR	0.97 (0.55–1.71)	1.40 (0.79–2.48)	2.27 (1.20–4.30)‡	1.96 (1.14–3.73)	1.83 (1.02–3.27)	3.08 (1.43–6.64)
OFFSPRING	n	17	23	10	29	13	10
	Random blood glucose (mmol/l)	5.23 ± 1.36	5.25 ± 1.26	7.80 ± 1.36	7.55 ± 1.15	5.00 ±1.20	5.78 ± 1.52
	Brain (% body weight)	4.04 ± 0.19	4.36 ± 0.16	4.34 ± 0.22	4.57 ± 0.15	4.61 ± 0.21	4.51 ± 0.24
	Heart (% body weight)	0.65 ± 0.04	0.70 ± 0.03	0.64 ± 0.05	0.66 ± 0.04	0.73 ± 0.06	0.59 ± 0.06
	Liver weight (% body weight) [##]	4.44 ± 0.21	4.92 ± 0.19	4.47 ± 0.25	4.63 ± 0.17	3.85 ± 0.25*†	4.80 ± 0.27
	n	12	12	12	12	12	12
	SOD (U/ml) [#]	0.13 ± 0.01	0.23 ± 0.01***††††	0.15 ± 0.01	0.15 ± 0.02	0.16 ± 0.02	0.14 ± 0.02
	8-OH-2dG (pg/ml)	53 ± 6	42 ± 5	50 ± 6	73 ± 7	53 ± 8	64 ± 7
	Free fatty acids (mmol/l)	0.24 ± 0.07	0.16 ± 0.07	0.27 ± 0.08	0.50 ± 0.08	0.41 ± 0.07	0.43 ± 0.08
	Total protein (g/dl)	2.48 ± 0.22	2.52 ± 0.20	2.88 ± 0.22	2.08 ± 0.29	1.92 ± 0.23	1.96 ± 0.29
	AST (U/l)	307 ± 67	435 ± 62	394 ± 76	376 ± 125	225 ±115	271 ± 136
	CK (U/l)	119 ± 32	114 ± 30	156 ± 35	104 ± 28	88 ± 26	89 ± 31
	LDH (U/l)	1672 ± 287	1850 ± 266	2033 ± 322	1675 ± 534	756 ± 527	632 ± 646

*p<0.05 and ***p<0.001 vs OxFO; ‡p<0.06, †p<0.05, ††p<0.01, and ††††p<0.0001 vs Control; [#]p<0.05 and [##]p<0.01 for a significant interaction between diet and gavage.

8-OH-2dG, DNA oxidation by-product 8-hydroxy-2-deoxy guanosine; AST, aspartate aminotransferase; CK, creatinine kinase; LDH, lactate dehydrogenase; and SOD, total superoxide dismutase.


T1
Lead-in diet – 10 days
T2
Pregnancy – 21 days
T3
Lactation – 21 days
T4
75
37
Control diet
38
High-fat diet
14
Control diet
Fresh fish oil gavage
11
Control diet
Oxidised fish oil gavage
12
Control diet
Water gavage (control)
13
High-fat diet
Fresh fish oil gavage
12
High-fat diet
Oxidised fish oil gavage
13
High-fat diet
Water gavage (control)
9 (127)
9 (115)
8 (94)
8 (95)
6 (72)
9 (123)
Control diet
Control diet
Control diet
High-fat diet
High-fat diet
High-fat diet
9
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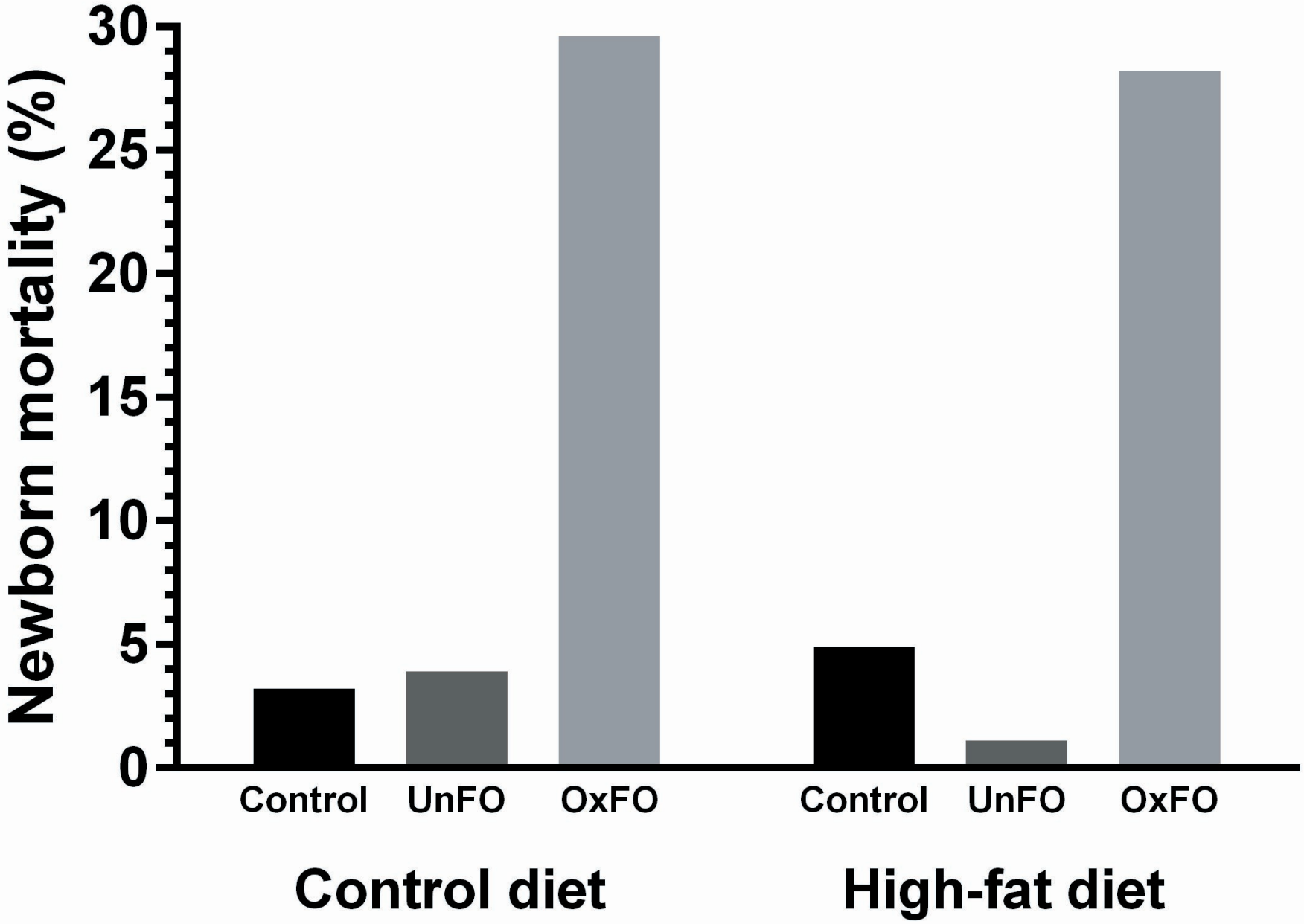
6
9


Newborn mortality (%)
30
25
20
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10
5
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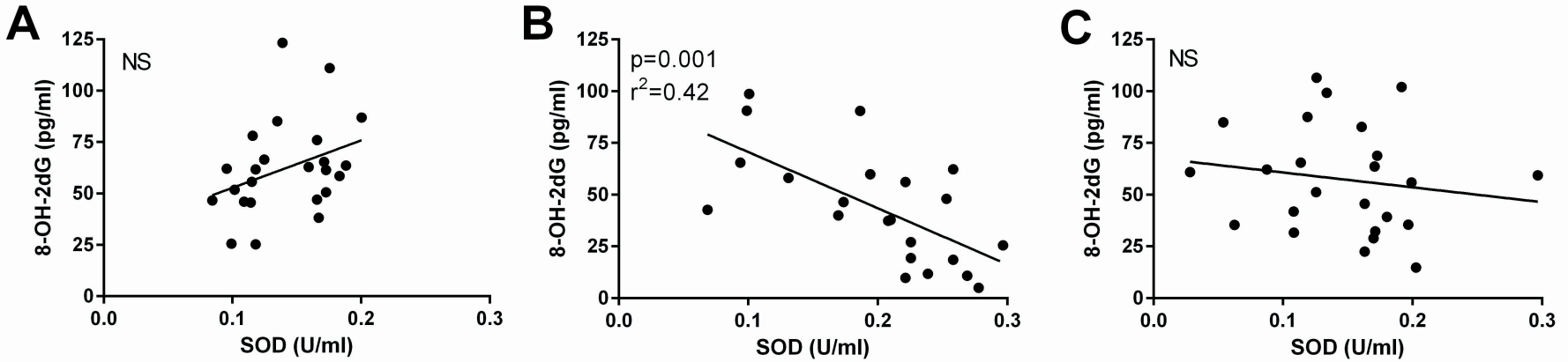
Control
UnFO
OxFO
Control
UnFO
OxFO
Control diet
High-fat diet


A
NS
8-OH-2dG (pg/ml)
125
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SOD (U/ml)
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p=0.001
r2=0.42
8-OH-2dG (pg/ml)
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SOD (U/ml)
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NS
8-OH-2dG (pg/ml)
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SOD (U/ml)