Omega-3 supplementation can restore glutathione levels and prevent oxidative damage caused by prenatal ethanol exposure

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Abstract

Prenatal ethanol exposure (PNEE) causes long-lasting deficits in brain structure and function. In this study, we have examined the effect of PNEE on antioxidant capacity and oxidative stress in the adult brain with particular focus on four brain regions known to be affected by ethanol: cerebellum, prefrontal cortex and hippocampus (cornu ammonis and dentate gyrus subregions). We have utilized a liquid diet model of fetal alcohol spectrum disorders that is supplied to pregnant Sprague-Dawley rats throughout gestation. To examine the therapeutic potential of omega-3 fatty acid supplementation, a subset of animals were provided with an omega-3-enriched diet from birth until adulthood to examine whether these fatty acids could ameliorate any deficits in antioxidant capacity that occurred due to PNEE. Our results showed that PNEE caused a long-lasting decrease in glutathione levels in all four brain regions analyzed that was accompanied by an increase in lipid peroxidation, a marker of oxidative damage. These results indicate that PNEE induces long-lasting changes in the antioxidant capacity of the brain, and this can lead to a state of oxidative stress. Postnatal omega-3 supplementation was able to increase glutathione levels and reduce lipid peroxidation in PNEE animals, partially reversing the effects of alcohol exposure, particularly in the dentate gyrus and the cerebellum. This is the first study where omega-3 supplementation has been shown to have a beneficial effect in PNEE, reducing oxidative stress and enhancing antioxidant capacity.

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1. Introduction

The consumption of alcohol during pregnancy can significantly damage the developing brain. Because the extent of the damage can vary due to the timing, frequency and volume of alcohol consumed, this damage can take the form of a number of disorders that are grouped under the term \textit{fetal alcohol spectrum disorders} (FASD). Prenatal ethanol exposure (PNEE) causes abnormal brain development and cell death in the central nervous system [1]. Maternal and genetic factors play a role, as do socioeconomic status, maternal nutrition and concomitant use of drugs of abuse [2,3]. Recent reports have estimated the prevalence of FASD in young school children in the

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United States and some Western European countries to be as high as 2%–5% [4], and it is thought that FASD is the most common cause of mental retardation and birth defects in the United States [5]. Although the mechanism(s) by which PNEE causes deleterious changes in the developing brain has not been fully elucidated, both in vivo and in vitro animal studies suggest that oxidative stress is likely to be involved [6–14] (for review, see Ref. [15]). Macromolecules that include lipids and proteins can be damaged by reactive oxygen species (ROS) [16]. This, in turn, can place the cell in a state of oxidative stress that results in altered cell signaling and, ultimately, cell death [17,18]. In addition, progressive oxidative damage may be linked to deficits in cognitive function and synaptic plasticity like those that are prevalent in FASD [19].

In healthy cells, antioxidants and free radical scavengers neutralize ROS and prevent cellular damage. If this process is impaired by ethanol, cells can be overcome by ROS, resulting in cell death [2]. Of particular importance for FASD is the fact that levels of antioxidants are much lower in the developing fetus than they are in mature cells [20,21], making developing neurons much more susceptible to the negative effects of oxidative stress [22]. In addition, early ethanol exposure can also produce a long-lasting impairement in the capacity of neurons in adult animals to handle ROS [10,15].

Omega-3 fatty acids are polyunsaturated fatty acids (PUFAs) that are found in high concentrations in neuronal membranes [23] and that have antioxidant properties [24]. Docosahexaenoic acid (DHA) is the most abundant omega-3 fatty acid in the brain, comprising 40% of the brain PUFAs [25]. DHA that is incorporated into the membrane can act to improve membrane fluidity, enhance synaptic transmission and increase receptor trafficking [26]. DHA can also activate energy-generating metabolic pathways that increase levels of growth factors including brain-derived neurotrophic factor and insulin growth factor-1 (IGF-1) [26]. These growth factors, in turn, can activate signaling cascades, through the tyrosine kinase B (TrkB) and IGF receptors, respectively, and enhance synaptic plasticity and transcription of genes involved in neuronal growth and development. Other metabolic effects of DHA include stimulating glucose utilization and mitochondrial function, further reducing the levels of free radicals in the brain [26].

Omega-3 fatty acid supplementation has been shown to improve the antioxidant status in both normal rodents and disease models where oxidative stress is increased [27,28]. Indeed, PNEE decreases brain concentrations of DHA [29,30], suggesting that omega-3 fatty acid levels in the brain of offspring born to ethanol drinking mothers are lower than normal. Since PNEE decreases brain concentrations of DHA [29] and omega-3 fatty acid supplementation has been shown to improve the antioxidant capacity of the brain in both healthy and disease animals [21,24,27], this study aimed to determine whether supplementation with an omega-3 fatty acid-enriched diet from birth is adequate to overcome this deficit and improve antioxidant status and consequently brain function in a rat model of PNEE.

2. Methods and materials

2.1. Animals and breeding

Four male (300–350 g) and 30 virgin female (250–275 g) Sprague-Dawley rats were obtained from Charles River Laboratories (Quebec, Canada) and housed in a colony room at the University of Victoria Animal Care Unit. Females were housed in pairs, and breeding males were housed individually in clear polycarbonate cages (46×24×20 cm) with Carefresh contact bedding (Absorption Corp., Bellingham, WA, USA). The room was maintained on a 12-hour light–dark cycle with constant humidity and temperature (22°C). Following an acclimation period in the unit for at least 1 week, females and males were housed together and a vaginal smear using 0.9% sodium chloride (NaCl) was performed at the beginning of each light cycle to determine pregnancy. The swab was examined on a microscope slide with an Olympus Microscope with a 10× objective (Olympus CX21, Center Valley, PA, USA) in order to detect the presence of sperm. If sperm was detected, this indicated gestational day 1 (GD1), and the female was immediately removed to a private container supplied with nesting material and placed on one of three prenatal diets. All animal procedures were performed in accordance with the University of Victoria and the Canadian Council for Animal Care policies.

2.3. Prenatal diets

On GD1, pregnant dams were randomly assigned to one of three prenatal diets:

1. Ethanol – ad libitum access to a liquid diet containing 35.5% ethanol-derived calories. Ethanol dams were gradually introduced to the liquid diet over a 3-day period (GDs 1–3). On GD1, one third ethanol diets was combined with two thirds pair-fed diet (see below); on GD2, two thirds ethanol diet was combined with one third pair-fed diet; and on GD3 (and all subsequent days of the pregnancy), full (three thirds) ethanol diets was supplied to the dam.

2. Pair-fed – a liquid diet with maltose-dextrin isocalorically substituted for the ethanol derived calories. This liquid diet was not provided if ad libitum. To control for stress and malnutrition, the pair-fed animals received the same amount of food in g/kg/day as their matched ethanol dams.

3. Ad libitum control – a regular chow diet (Lab Diets 5001; LabDiet, Richmond, IN, USA) that animals had ad libitum access to throughout pregnancy.

All diets were given to the animals 2 hours prior to the beginning of the dark phase each day of the pregnancy. This was done to ensure that there were no shifts in the circadian rhythm [31]. When liquid diet bottles were replaced, the bottle from the previous day was weighed to determine the amount of liquid consumed each day. Liquid diets were obtained from Dyets (Bethlehem, PA, USA) where they are sold as Weinberg/Keiger high protein liquid diet-control (no. 710109) for the pair-fed diet and Weinberg/Keiger high protein liquid diet-experimental (no. 710324) for the ethanol diet. These liquid diets have been nutritionally fortified to ensure that adequate nutrition is provided to the pregnant rats [32].

2.4. Blood alcohol concentration assay

For all ethanol-fed dams, a single tail blood sample was obtained on GD15, approximately 2 hours after beginning of the dark phase. Blood was collected in a microcentrifuge tube (1 ml) and allowed to clot overnight at 4°C. Samples were centrifuged the following day at 3000g for 10 min, and the serum (supernatant) was then stored at −20°C until analyzed. Analysis of blood alcohol concentration (BAC) was determined using the Analox Alcohol Analyzer (Model AMI; Analox Instruments, Lunenburg, MA, USA) and expressed as mg/dl of serum.

2.5. Postnatal diets

Half the dams in each group were switched to regular rat chow (Lab Diets 5001; Table 1) on GD21, while the other half was placed on an omega-3–enriched powder diet (kindly supplied by Dr. Sheila Innis, University of British Columbia, Canada; Table 1). The omega-3 diet was supplied in a glass jar in the bottom of the cage and was replaced every day to prevent oxidation of the omega-3 fatty acids in the diet. The dams stayed on the respective diets throughout the suckling period, and upon weaning, the pups were continued on the same diet as their mother.

2.6. Litters and weaning

The dams and pups were not disturbed for the first 24–36 hours postpartum to facilitate bonding. Litters were culled to 10 pups (4 females and 6 males, or 6 females and 4 males where possible) on postnatal day (PND) 2, and all animals were weighed. The pups were weaned at 22 days of age and housed in pairs (based on sex) in standard caging. Each pup was maintained on either the regular chow diet or the omega-3–enriched diet, depending on the diet their mother was maintained on during the suckling period. Animals were given ad libitum access to the diet from PNDs 22 to 60. The omega-3 diet was replaced daily to avoid fatty acid oxidation.

2.7. Tissue preparation

In this study, we examined changes in oxidative stress parameters in the cerebellum (CB), prefrontal cortex (PFC), and the cornu ammonis (CA) and dentate gyrus (DG) subregions of the hippocampus. At PND 60, animals were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL, USA) and quickly decapitated, and

<table>
<thead>
<tr>
<th>Fat</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omega-3 fatty acids</td>
<td>0.29%</td>
<td>34.2% (24.6% DHA)</td>
</tr>
<tr>
<td>Omega-6 fatty acids</td>
<td>1.22%</td>
<td>4.1%</td>
</tr>
</tbody>
</table>
the brains were dissected from the skull and placed in ice-cold tris-buffered saline (0.1 M, pH 7.4). The CB and PFC were dissected off from both hemispheres, and the CA and DG subregions of the hippocampus were crudely microdissected following the procedure described by Hagihara et al. [33]. Briefly, each brain was cut along the longitudinal fissure of the cerebrum, and the midbrain, hindbrain and CB were removed. With the cerebral hemisphere medial side facing up, the diencephalon was removed, which exposed the medial side of the hippocampus and allowed for the visualization of the DG, as the boundary between the DG and CA subsections is identifiable with the naked eye. A surgical spatula was used to separate the DG from the CA region by sliding the spatula along the septotemporal axis of the hippocampus.

Samples that were used to determine the activity of endogenous antioxidant enzymes were sonicated four times for 5 s in 20 mM 1,4-dihydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4). Lysates were then centrifuged at 15,000 g for 30 min at 4°C, and the supernatants were stored at –80°C until processing. Samples used to determine the total levels of glutathione (GSH) were homogenized in cooled 0.5 M perchloric acid. The homogenates were centrifuged at 15,000 g for 2 min, and the supernatant was separated and neutralized in potassium phosphate buffer (phosphate-buffered saline, 0.1 M, pH 7.4). The samples were immediately processed as described below.

The total protein content of all the samples was determined by the bicinchoninic acid (BCA) method using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) and following the manufacturer’s instructions.

2.8. Analysis of antioxidant status and markers of oxidative damage

The levels of the endogenous antioxidant GSH, lipid peroxidation (thiobarbituric acid reactive substances, or TBARS) and protein carbonyls, as well as the activity of superoxide dismutase (SOD) and catalase (CAT) were measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). With regard to the activity of the endogenous antioxidants that utilize GSH as a cofactor, GSH peroxidase (GPx), GSH reductase (GR) and GSH-S-transferase (GST) were determined by spectrophotometry (Ultr spec 3000; Pharmacia Biotech, Cambridge, UK).

2.8.1. Determination of GSH levels

GSH is the major antioxidant in the brain. It acts as a nzenzymatic antioxidant, is a cofactor in other antioxidant reactions (GPx and GST both use GSH as a cofactor), and is also involved in many other processes such as cytochrome storage and redox buffering and has neuromodulatory functions [34,35].

Total GSH (GSH-t) levels, comprising both of its reduced (GSH, which normally constitutes more than 95% of GSH-t) and oxidized (GSGS) forms, was measured by the reaction of the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) – GR recycling assay [36]. The sulfhydryl group of GSH reacts with DTNB and produces a yellow colored 5-thio-2-dithiobis(2-nitrobenzoic acid) (TNB). GSTNB, the mixed disulfide formed between GSH and TNB, is reduced by GR to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which, in turn, is directly proportional to the concentration of GSH measured at 412 nm. GSH-t levels were expressed in μmol/g wet tissue.

2.8.2. GPx activity

GPx is an antioxidant enzyme that is responsible for detoxifying hydrogen peroxide (H₂O₂) by converting it to water. When H₂O₂ is reduced by GPx, GSH acts as an electron donor in the reaction [37]. GPx activity was measured indirectly by a coupled reaction with GR, using the method described by Wendel [38]. In this assay, GPx catalyzes the reduction of organic peroxide, oxidizing GSH to form GSSG. GSSG is recycled back to GSH by GR with the concomitant conversion of NADPH into NADP⁺. The reaction was performed at 25°C and pH 8.0 in the presence of saturating concentrations of exogenous GSH and NADPH and was started by adding an organic peroxide, tert-butyl hydroperoxide. The rate of decrease in absorbance at 340 nm is directly proportional to the GPx activity in the sample. GPx activity was expressed in nmol/mg protein.

2.8.3. GR activity

GR is a homodimeric flavoprotein that catalyzes the reduction of GSSG using NADPH as a cofactor. The enzyme is a major component of cellular defense mechanisms against oxidative injury and plays an essential role in the prevention of oxidative damage by maintaining GSH levels [39].

GR activity in brain homogenates was determined by the method described by Carlberg and Mannervik [40]. This assay is based on the reduction of GSSG by NADPH, which is catalyzed by GR. The concomitant oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm that is directly proportional to the GR activity in the sample. The assay was performed in the presence of saturating concentrations of the enzyme substrates (GSSG and NADPH). GR activity was expressed in nmol/mg protein.

2.8.4. GST activity

GST is an enzyme found predominantly in astrocytes [41] that catalyzes the conjugation of electrophilic substances to GSH, thus facilitating their transport out of the cell [42]. GST is also the enzyme responsible for converting 4-hydroxynonenal (one of the products of lipid peroxidation) to the GSH-HNE adduct, reducing its damaging ability [43].

Brain GST activity was measured essentially as described by Habig and Jakoby [44] using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. CDNB is not specific for any particular GST isoenzyme, being able to react with a broad range of GST isoenzymes. The assay was conducted by monitoring the appearance of the conjugated complex of CDNB and GSH, which results in an increase in the absorbance at 340 nm. GST activity was expressed in nmol/mg protein.

2.8.5. SOD activity

SODs are a class of metalloprotein enzymes responsible for inactivating super oxide radicals by converting them to H₂O₂ and molecular oxygen [45].

Brain SOD activity was determined using a SOD activity assay kit (BioVision Life Science, San Francisco, CA, USA) following the manufacturer’s instructions and was measured at 450 nm. SOD activity was expressed in U/mg protein, where 1 U equals the amount of SOD needed to exhibit 50% dissipation of the superoxide radical.

2.8.6. CAT activity

CAT is an important peroxisomal heme-containing enzyme that prevents H₂O₂ build up in the cell by catalyzing its decomposition to water and oxygen [45].

Brain CAT activity was measured using a CAT activity assay kit (BioVision Life Science) according to the manufacturer’s directions. In the assay, CAT first reacts with H₂O₂ to produce water and oxygen, and any unconverted H₂O₂ reacts with the OxIR probe (BioVision Life Science) to produce a product, which can be measured at 570 nm. CAT activity was expressed in U/mg protein, where 1 U equals the amount of CAT that decomposes 1 μmol of H₂O₂ per minute.

2.8.7. Lipid peroxidation levels

As an index of lipid peroxidation, the formation of TBARS [46] was measured in homogenates from the different brain regions, using the commercially available TBARS assay kit (Biocassay Systems, Hayward, CA, USA) and following the manufacturer’s instructions. The amount of malondialdehyde (MDA) produced was determined spectrophotometrically at 525 nm. The results were expressed in μM MDA/mg protein.

2.8.8. Protein carbonyl levels

Protein oxidation occurs when proteins are covalently modified either by ROS (direct) or by reacting with by-products of oxidative stress (indirect) [47]. Protein carbonyls are derivatives of amino acids such as Pro, Arg, Lys and Thr, which are chemically stable and can be used as markers of oxidative stress.

The levels of carbonyl groups were determined based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) and using a protein carbonyl assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) that utilizes the DNPH reaction to measure protein carbonyl content. The amount of protein-hydroxylamine produced was determined spectrophotometrically at an absorbance between 360 and 380 nm. The results were expressed in nmol/mg protein.

2.9. Statistical analyses

Statistical analysis was performed using the Statistica 7.1 analytical software (StatSoft Inc., Tulsa, OK, USA). All data are presented as mean±standard error of the mean (S.E.M.). A one-way analysis of variance (ANOVA) was used to determine the effect of prenatal treatment on weight gain across pregnancy and litter size. A repeated-measures ANOVA for prenatal diet (ethanol, pair-fed, ad libitum) and postnatal diet (omega-3, regular diet) was used for developmental data of pup weight taken on PNDs 2, 22 and 60. Because there were no significant effects of sex, data from male and female were combined and a two-way ANOVA for prenatal treatment and diet was performed for each of the enzymes and oxidative stress assays. Post hoc analyses were conducted using Tukey’s test. A P value ≤0.05 was considered statistically significant.

3. Results

3.1. Developmental data

Weight data were taken from the dams on GDs 1, 8, 15 and 21. The percentage weight gain over the course of pregnancy did not differ between prenatal treatment groups [F(2,30)=2.89, P=0.07] (Table 2). Interestingly, there was a significant effect of litter size between conditions [F(2,38)=6.21, P<0.01]. Post hoc analyses revealed that ad libitum control dams had significantly fewer pups when compared to pair-fed (P<0.01) and ethanol-exposed animals (P<0.05; Table 2). The BAC levels were measured 2 h after the dark cycle commenced on GD 15 of pregnancy. The mean BAC level was 135.40±7.54 mg/dl.
Offspring weight was measured on PNDs 2, 22 (weaning) and 60 to determine whether prenatal treatment or postnatal diet altered offspring weight gain (Table 3). A repeated-measures ANOVA revealed that there was a significant main effect of treatment \([F(6,60)=10.0, P<.001]\): at PND 2, ad libitum weighed more than pair-fed \((P<.001)\) or ethanol-exposed animals \((P<.001)\); at PND 22, ad libitum weighed less than pair-fed \((P<.001)\) or ethanol-exposed animals \((P<.001)\), with no treatment differences at PND 60. There was also a significant main effect of sex \([F(3,30)=75.2, P<.001]\): at PND 2 \((P<.05)\) and at PND 60 \((P<.001)\), males weighed more than females, but no differences were observed at PND 22. Postnatal diet did not influence the weight of the offspring at any time points measured (Table 3).

### 3.2. GSH-t levels

PNEE significantly reduced the levels of GSH-t in all brain regions analyzed, DG (Fig. 1A): \([F(2,91)=71.8, P<.001]\), with DG GSH-t levels being significantly reduced in ethanol-exposed rats when compared with their ad libitum controls \((P<.001)\) and pair-fed animals \((P<.001)\); CA (Fig. 1B): \([F(2,85)=52.2, P<.001]\), with CA GSH-t levels being significantly reduced in ethanol-exposed rats when compared to both ad libitum controls \((P<.001)\) and pair-fed animals \((P<.001)\); CB (Fig. 1C): \([F(2,91)=31.4, P<.001]\), with cerebellar GSH-t levels being significantly reduced in ethanol-exposed rats when compared to ad libitum control \((P<.001)\) and pair-fed animals \((P<.001)\); PFC (Fig. 1D): \([F(2,81)=40.5, P<.001]\), with PFC GSH-t levels being reduced as compared to ad libitum animals \((P<.001)\).

A significant effect of diet was observed in all brain regions: DG \([F(1,91)=25.3, P<.001]\), CA \([F(1,85)=20.4, P<.001]\), CB \([F(1,91)=25.0, P<.001]\), and PFC \([F(1,81)=8.3, P<.01]\) (Fig. 1). However, omega-3 supplementation was only able to significantly reverse the ethanol-induced decrease in GSH-t levels in the DG \((P<.05)\) and CB \((P<.01)\) (Fig. 1). In these regions, the level of GSH-t following omega-3 supplementation was no longer significantly different from pair-fed animals.

Our results suggest that PNEE has a detrimental and long-term effect on GSH-t levels in the brain. This effect is most pronounced in the DG subregion of the hippocampal formation and the CB, but is also seen in the PFC and the CA subregion of the hippocampus (Fig. 1).

### 3.3. Antioxidant enzymes

Interestingly, no changes in the activity of the antioxidant enzymes related with GSH cycle Gpx, GR, GST, or in the enzymes SOD and CAT were seen following PNEE (Tables 4 and 5). This indicates that in this FASD model, ethanol has a very specific effect on GSH levels without reducing the principal antioxidants enzymes in the brain. Omega-3 supplementation had also no effect on the activity of these enzymes (Tables 4 and 5). Specific statistical changes are noted in Tables 4 and 5.

### 3.4. Lipid peroxidation

PNEE increased lipid peroxidation in the DG (Fig. 2A) \([F(2,89)=3.11, P<.05]\) when compared to both ad libitum controls \((P<.01)\) and pair-fed animals \((P<.01)\) and in the PFC (Fig. 2D) \([F(2,89)=3.70, P<.05]\) when compared to pair-fed controls \((P<.01)\) (Fig. 2). However, in these two brain regions, omega-3 supplementation was able to reverse the ethanol-induced increase in lipid peroxidation \([DG (F[1,89])=10.4, P<.01]; PFC (F[1,89])=3.9, P<.05]\). Interestingly, omega-3 supplementation did not reduce lipid peroxidation in ad libitum control animals (Fig. 2) and, in fact, increased this measure of oxidative damage in the CA subregion \([F(1,89)=7.9346, P<.01]\).

### 3.5. Protein carbonyls

There was no significant effect of PNEE on protein carbonyl formation in any of the brain regions examined (Fig. 3). Furthermore, omega-3 supplementation also had no effect on the levels of protein carbonyls in any of the regions except the CA, where a significant effect of diet \([F(1,89)=6.398, P<.05]\) was observed. However, post hoc analyses failed to reveal any statistically significant differences among experimental groups (Fig. 3). In the CB and CA regions, omega-3 supplementation induced a nonsignificant trend toward a decrease in the levels of protein carbonyls in ethanol-exposed animals.

### 4. Discussion

The results of this study demonstrate that omega-3 fatty acid supplementation was able to overcome long-lasting deficits in GSH levels caused by PNEE, suggesting that omega-3 supplementation may be a viable treatment option for preventing oxidative stress in individuals diagnosed with FASD.

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**Table 2**

Gestational data for ad libitum, pair-fed and ethanol dams

<table>
<thead>
<tr>
<th></th>
<th>Ad libitum</th>
<th>Pair-fed</th>
<th>Ethanol-exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain during pregnancy (%)</td>
<td>52.7±4.6</td>
<td>43.7±2.44</td>
<td>41.3±3.19</td>
</tr>
<tr>
<td>Number of pups per litter</td>
<td>14±0.76*</td>
<td>17.4±0.7</td>
<td>16.4±0.56</td>
</tr>
</tbody>
</table>

All dams had comparable weight gain over the course of their pregnancies. Ad libitum dams had significantly less pups per litter than ethanol and pair-fed dams. * P<.05 compared to pair-fed and ethanol-exposed animals.

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**Table 3**

Weight of the offspring from birth to adulthood

<table>
<thead>
<tr>
<th>Offspring weight</th>
<th>Ad libitum</th>
<th>Pair-fed</th>
<th>Ethanol-exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regular diet</td>
<td>Omega-3 diet</td>
<td>Regular diet</td>
</tr>
<tr>
<td>Male PND 2</td>
<td>8.8±0.5***</td>
<td>9.0±0.4***</td>
<td>6.6±0.5</td>
</tr>
<tr>
<td>Male PND 22</td>
<td>32±3.4***</td>
<td>45.8±4.2***</td>
<td>48.4±4.1</td>
</tr>
<tr>
<td>Male PND 60</td>
<td>360.8±3.0</td>
<td>313.2±5.1</td>
<td>354.5±9.8</td>
</tr>
<tr>
<td>Female PND 2</td>
<td>8.0±0.4***</td>
<td>7.9±0.2***</td>
<td>6.4±0.4</td>
</tr>
<tr>
<td>Female PND 22</td>
<td>49.6±3.1***</td>
<td>43.4±4.2***</td>
<td>46.4±4.2</td>
</tr>
<tr>
<td>Female PND 60***</td>
<td>242.0±4.3</td>
<td>229.8±4.6</td>
<td>215.5±18.4</td>
</tr>
</tbody>
</table>

*** P<.001 vs. pair-fed and ethanol-exposed.
* P<.05 vs. males.
*** P<.001 vs. males.
4.1. Omega-3 supplementation can restore GSH levels following PNEE

GSH, the major nonenzymatic antioxidant in the brain [48], was significantly decreased in the four brain regions analyzed of adult rats that were exposed to ethanol during embryonic development (Fig. 1). The deficits in GSH may account, at least in part, for the increase in oxidative stress that was observed following PNEE, as indicated by the elevated levels of lipid peroxidation (Fig. 2). Interestingly, PNEE animals that received omega-3 supplementation from birth until adulthood did not show deficits in GSH (Fig. 1), showing that omega-3 supplementation can restore GSH levels and prevent oxidative stress in the adult brain. These results are in agreement with previous studies that have shown that omega-3 supplementation can increase GSH levels in both normal brains [49] and those hampered by different disease states [50,51]. We [14] and others [10] have previously shown that ethanol exposure during the perinatal period leads to a long-lasting depletion of brain GSH levels. In the present study, we also found that exposure to ethanol during gestation results in a pronounced reduction of brain GSH levels in adulthood, illustrating the long-lasting and damaging effects of PNEE. In previous studies, a gavage model of PNEE was used to examine oxidative stress later in life in the adult brain [10,14].

Table 4

The effect of PNEE and subsequent omega-3 fatty acid supplementation on the activities of GSH-related enzymes in different brain regions of PND 60 adult rats

<table>
<thead>
<tr>
<th>Prenatal Treatment</th>
<th>GPx (nmol/mg protein)</th>
<th>GR (nmol/mg protein)</th>
<th>GST (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regular chow diet</td>
<td>Omega-3-enriched diet</td>
<td>Regular chow diet</td>
</tr>
<tr>
<td>DG</td>
<td>Ad libitum</td>
<td>15.7±1.3</td>
<td>18.0±1.5</td>
</tr>
<tr>
<td></td>
<td>Pair-fed</td>
<td>17.3±1.4</td>
<td>19.0±1.3</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>16.9±1.7</td>
<td>17.1±1.3</td>
</tr>
<tr>
<td>CA</td>
<td>Ad libitum</td>
<td>18.3±1.1</td>
<td>17.0±1.3</td>
</tr>
<tr>
<td></td>
<td>Pair-fed</td>
<td>21.2±2.1</td>
<td>22.3±1.9</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>20.5±2.1</td>
<td>21.8±1.1</td>
</tr>
<tr>
<td>PFC</td>
<td>Ad libitum</td>
<td>21.7±3.9</td>
<td>19.3±2.1</td>
</tr>
<tr>
<td></td>
<td>Pair-fed</td>
<td>19.7±1.4</td>
<td>27.0±1.8</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>19.0±2.4</td>
<td>18.2±1.4</td>
</tr>
<tr>
<td>CB</td>
<td>Ad libitum</td>
<td>39.7±5.7</td>
<td>46.7±6.0</td>
</tr>
<tr>
<td></td>
<td>Pair-fed</td>
<td>28.5±3.3</td>
<td>45.3±4.7</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>29.6±3.6</td>
<td>37.0±4.7</td>
</tr>
</tbody>
</table>

Ad libitum regular diet: n=15, ad libitum omega-3 diet: n=15; ethanol regular diet: n=16, ethanol omega-3 diet: n=20; pair-fed regular diet: n=16, pair-fed omega-3 diet: n=15.

* P<.05 compared to ad libitum control.

** P<.05 compared to regular chow diet.

Fig. 1. The effect of PNEE and subsequent omega-3 fatty acid supplementation on GSH levels in different brain regions of PND60 adult rats. PNEE decreased GSH levels in all brain regions examined. Omega-3 fatty acid treatment increased GSH levels in both ad libitum and PNEE animals. Ad libitum regular diet and ad libitum omega-3 diet; n=15, pair-fed regular diet; n=16, pair-fed omega-3 diet; n=14, ethanol regular diet; n=14, ethanol omega-3 diet; n=22. Data are presented as means±S.E.M. See text for further statistical details. **P<.001.
procedure typically produces higher BAC levels and a more severe phenotype of FASD [52]. Accordingly, in these two studies, deficits were also observed in the activity of the various endogenous antioxidant enzymes. In the current study, using a two-trimester liquid diet model with lower BAC levels, a decrease in the levels of GSH can still be observed. This suggests that the first step in the cascade of ethanol-induced oxidative stress corresponds to a depletion of GSH, which is apparent even with milder alcohol intoxication. With greater levels of intoxication (as seen in the studies utilizing the gavage model [53]), GSH depletion may have more severe consequences leading to alterations in the activities of the antioxidant enzymes that use GSH as a cofactor.

In this study, the two brain regions that were most affected by PNEE were the DG of the hippocampus and the CB. A similarity between these brain regions is that they are both primarily populated by granule cells. Interestingly, granule cells do not develop until the third trimester equivalent (PNDs 1–10) in the rat [54]. As such, the development of these cells occurred after the animals have been exposed to ethanol, indicating that ethanol may have damaged the population of granule cell precursors, leading to lower GSH levels in

![Fig. 2. The effect of PNEE and subsequent omega-3 fatty acid supplementation on the level of lipid peroxidation in different brain regions from PND 60 adult rats. Ad libitum regular diet: n = 15, ad libitum omega-3 diet: n = 15; ethanol regular diet: n = 16, ethanol omega-3 diet: n = 20; pair-fed regular diet: n = 16, pair-fed omega-3 diet: n = 15. * P<.05 compared to ad libitum control. # P<.05 compared to regular chow diet.](Image)

**Table 5**
The effect of PNEE and subsequent omega-3 fatty acid supplementation on SOD and CAT activities in different brain regions from PND 60 adult rats

<table>
<thead>
<tr>
<th>Prenatal treatment</th>
<th>SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regular chow diet</td>
<td>Omega-3-enriched diet</td>
</tr>
<tr>
<td>DG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>0.510±0.034</td>
<td>0.509±0.045</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>0.489±0.032</td>
<td>0.546±0.014</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.464±0.026</td>
<td>0.543±0.032</td>
</tr>
<tr>
<td>CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>0.581±0.029</td>
<td>0.550±0.033</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>0.449±0.034*</td>
<td>0.515±0.030*</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.458±0.027</td>
<td>0.560±0.031</td>
</tr>
<tr>
<td>PFC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>0.401±0.029</td>
<td>0.424±0.027</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>0.342±0.023</td>
<td>0.355±0.028</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.320±0.031</td>
<td>0.397±0.031</td>
</tr>
<tr>
<td>CB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>0.364±0.022</td>
<td>0.394±0.034</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>0.362±0.036</td>
<td>0.320±0.018</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.331±0.025</td>
<td>0.407±0.029</td>
</tr>
</tbody>
</table>

SOD-1U=amount of SOD needed to exhibit 50% dismutation of the superoxide radical; CAT-1U, amount of CAT that decomposes 1 μmol of H₂O₂ per minute. Ad libitum regular diet: n = 15, ad libitum omega-3 diet: n = 15; ethanol regular diet: n = 16, ethanol omega-3 diet: n = 20; pair-fed regular diet: n = 16, pair-fed omega-3 diet: n = 15.

* P<.05 compared to ad libitum control.

# P<.05 compared to regular chow diet.

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but have failed to investigate whether such antioxidants would still have tested the effects of other antioxidants such as silymarin when administered concomitantly with ethanol, have failed to investigate whether such antioxidants would still be beneficial when given after the initial insult. While providing supplementation concomitantly with ethanol exposure shows beneficial results, it may have little relevance in a clinical setting where alcohol abusing mothers may be unlikely to take supplementation. Providing supplementation following the ethanol insult, however, could be a viable treatment option for children born to alcohol abusing mothers.

4.2. Effects of PNEE and omega-3 supplementation on the activity of antioxidant enzymes

PNEE did not alter the activity of any of the antioxidant enzymes measured in this study and thus it is not surprising that omega-3 supplementation also did not affect antioxidant activity. These results are in contrast with other studies that have observed alterations in the activities of certain antioxidant enzymes following PNEE [10,14,65,66]. However, these studies used different models of ethanol exposure (i.e., intragastric intubation and vapor inhalation) and different exposure periods (single-day exposure during gestation or gavage throughout pregnancy), and the age of analysis varied (PNDS 3–21, adult), making comparisons difficult.

The fact that none of the antioxidant enzymes in the brain were affected by omega-3 supplementation could mean that these enzymes are already active at maximal capacity, or it could indicate the omega-3 supplementation has a very specific effect on GSH in the brain. Indeed, GSH is the major antioxidant in the brain and is a cofactor in many antioxidant enzyme reactions, so a deficit in GSH may be much more relevant than changes in any single enzyme. Alternatively, these results may also indicate that GSH plays a more specific role in the defence against oxidative stress than the enzymatic antioxidants. In fact, no major abnormalities or increased sensitivity to hypoxia have been observed in transgenic mice deficient in GPx [67]. Furthermore, overexpressing GPx in the hippocampus following traumatic brain injury did not rescue neurogenesis [68].
4.3. Effects of PNEE and omega-3 supplementation on oxidative damage

In this study, we observed an increase in lipid peroxidation in two areas of the brain with PNEE: the DG and the PFC. These results are in agreement with previous studies that have examined lipid peroxidation in the adult brain following PNEE [10,14,69]. Interestingly, in our previous study, we found an increase in lipid peroxidation in the CB and CA regions as well as the DG [14]. This differs from the present study where no changes were seen in the CA region and the CB. This is particularly interesting in the CB since, as discussed above (Section 4.1), GSH levels were notably decreased in this region after PNEE. While the reasons for these discrepancies are not clear, one possibility is the period of ethanol exposure. In the gavage model utilized previously [14], ethanol was also administered during the third trimester of development (PNDs 4–10), whereas the liquid diet is only administered during gestation (first and second trimester equivalent). During the third trimester, large amounts of growth are occurring in both the CB and hippocampus, so ethanol exposure during this period may cause increased lipid peroxidation in these regions [55].

Importantly, the increase in lipid peroxidation was prevented by supplementation with an omega-3-enriched diet (Fig. 2), which is in agreement with previous studies that have noted decreased lipid peroxidation in the brain following omega-3 supplementation [21,24,70]. For example, omega-3 supplementation was shown to prevent formaldehyde-induced increase in lipid peroxidation in the PFC [27].

Protein carbonyl formation, a measure of oxidative damage to cellular proteins, was less affected by PNEE and omega-3 supplementation. It is still unclear why this may be the case, but lipid peroxidation is usually one of the initial steps in oxidative cascades, and the products of lipid peroxidation (such as MDA) are known to oxidize proteins, leading to the formation of carbonyl groups [71]. In previous studies, an increase in protein oxidation with PNEE has been detected [14,64]. However, as discussed above, differences in the models of PNEE used, the periods of exposure and the age at the time of analysis may underlie these discrepancies.

4.4. Conclusions

In conclusion, we have demonstrated the protective role of omega-3 fatty acids in preventing oxidative stress in the brains of rats that were exposed to ethanol during gestation. We showed that omega-3 fatty acid supplementation, given after ethanol exposure from birth until adulthood, can partially restore GSH levels and reduce PNEE-induced lipid peroxidation (Fig. 4). Therefore, we suggest that omega-3 fatty acids may be a useful therapeutic strategy for the treatment of some of the symptoms associated with FASD.

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References


