EFFECTS OF DOCOSAHEXAENOIC ACID SUPPLEMENTATION ON GLOBAL GENE EXPRESSION IN FETAL BRAIN IN RATS EXPOSED TO ETHANOL PRENATALLY

by

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Abstract

Prenatal alcohol exposure is known to change genes involved in signal transduction and neurological system processes, thus affecting normal fetal brain development. Omega-3 docosahexaenoic acid (DHA), a major component of brain, is known to be decreased in brain with alcohol consumption justifying its provision is necessary with alcohol exposure during pregnancy. This study was planned to examine if DHA supplementation impacts on global gene expressions in fetal brain exposed to ethanol during entire gestation period. Upon pregnancy, Sprague Dawley rats were raised on a control diet (Cont), Cont+Ethanol (EtOH, 6g/kg body weight, via gavage), or EtOH+DHA supplementation (1.4%, w/w, total fat) during gestational period. The diet was semi-purified nutritionally complete-energy dense (4.3 Kcal/g diet), reflecting typical intake of Canadians pregnant women. The global gene expression in the fetal brain (n=5 each group, 1-2 from each dam) in gestational day 20 was analysed by microarray and real-time polymerase chain reaction (qRT-PCR) for validation. The protein expression was analysed by Western blot. No significant differences were identified in the fetal brain and body weights among the groups. Microarray analysis revealed that none of the transcripts were significantly altered in fetus brain (Log{2}(fold change) ≤ ± 2, and P > 0.05). qRT-PCR, agreed with microarray data in fold changes (FC<2 or FC>0.5). DHA supplementation significantly (p<0.05) decreased the increased expression of PCDHB6 with EtOH, to the levels in the Cont group. EtOH also decreased WDR92 expression regardless of a DHA provision in comparison to the Cont. EtOH+DHA significantly decreased the expression of NFIA, NEUROD1, GRIN1 and WDR92 in comparison to the Cont. Western blot revealed that none of the proteins expressed significantly different among the groups. These findings indicate that the DHA supplementation have minor impacts on global gene expressions in the fetal brain exposed to ethanol during entire gestation period. It is speculated that the nutrient dense diet provided in this study may have mitigated the effects of ethanol. Future studies can be aimed at studying global gene expression with low vs high energy dense diet.
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<tbody>
<tr>
<td>FASD</td>
<td>Fetal alcohol spectrum disorder</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>FAS</td>
<td>Fetal alcohol syndrome</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational day</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>MEOS</td>
<td>Microsomal ethanol oxidizing system</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ADH</td>
<td>Antidiuretic hormone</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ARBD</td>
<td>Alcohol-related birth defects</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>Igf1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>Efemp1</td>
<td>EGF containing fibulin like extracellular matrix protein 1</td>
</tr>
<tr>
<td>Klf10</td>
<td>Kruppel like factor 10</td>
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<td>Edil3</td>
<td>EGF like repeats and discoidin domains 3</td>
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<tr>
<td>Sox5</td>
<td>SRY (Sex determining region Y)-box 5</td>
</tr>
<tr>
<td>Bhlhe22</td>
<td>Basic helix-loop-helix family member E22</td>
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ADH  Alcohol dehydrogenase
GH  Growth hormone
SREBP  Sterol regulatory element-binding proteins
PPAR  Peroxisome proliferator-activated receptors
RXR  Retinoid X receptor
BW  Body weight
EtOH  Ethanol
Cont  Control
PCDHB6  Protocadherin beta 6
NFIA  Nuclear factor I A
NEUROD1  Neuronal differentiation 1
FGF1  Fibroblast growth factor 1
WDR92  WD repeat domain 92
GRIN1  Glutamate ionotropic receptor NMDA type subunit 1
TAF1D  TATA-box binding protein associated factor, RNA polymerase I subunit D
OLR101  Olfactory receptor 101
CLDN12  Claudin 12
PPP1R14C  Protein phosphatase 1 regulatory inhibitor subunit 14C
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
qRT-PCR  Quantitative reverse transcription polymerase chain reaction
ED  Embryonic day
PUFA  Polyunsaturated fatty acids
SFA  Saturated fatty acids
ARASCO  Arachidonic single cell oil
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>SG</td>
<td>SYBR green</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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CHAPTER I: INTRODUCTION

Introduction

Despite strict warnings regarding the negative effects of alcohol consumption during pregnancy by different social media presences, the health ministries and other such educational awareness programs, alcohol consumption rates during pregnancy are high. It has been estimated that 3.3% of pregnant women continue to consume alcohol chronically (defined as 7 drinks/week) or binge drink (when 5 drinks are consumed per occasion) (Burd et al., 2004).

Alcohol consumption during pregnancy has many teratogenic effects on the unborn child which could lead to permanent lifetime effects even after birth. These detrimental effects are collectively termed “fetal alcohol spectrum disorder (FASD)”. The most obvious signs of FASD are seen in the facial characteristics of patients with fetal alcohol syndrome (FAD), but the most devastating consequences are the ones related to brain damage (Warren et al., 2011). Alcohol exposure has been shown to change the expression of genes involved in signal transduction in the neurological system of the fetal brain (Mandal et al., 2015).

It has also been observed that the threat of neuro-development in FASD can be alleviated by consumption of omega-3 fatty acids, especially docosahexaenoic acids (DHA) by the mother during pregnancy. DHA is a membrane structural component of brain and its deficiency impairs phosphatidylserine synthesis, which results in a decrease of neuronal development and survival, therefore it leads to impaired cognitive and overall central nervous system (CNS) development (Horrobin, 1987; Sadli et al., 2012). How DHA influences genes associated with a fetal brain exposed to alcohol has not been studied yet.

The area of focus for this study is to investigate whether DHA supplementation during pregnancy affects global gene expressions in fetal brains with prenatal ethanol exposure.
Brain development

The cell precursors of the brain and spinal cord or the central nervous system (CNS), begin to develop early in embryogenesis through the process called neurulation. The central rod, which characterizes the primitive axis of the embryo (namely the notochord) is meant to be integrated into the vertebral system. In the case of humans, the ectodermal tissue is induced by the notochord to develop the neural plate at around two weeks of gestation (Morreale De Escobar et al.). The CNS is actually formed from this ectodermal tissue. On the 18\textsuperscript{th} day of gestation, a neural groove is formed due to the invagination of the neural plate along its central axis. This groove has neural folds on both sides. These neural folds start to budge and fuse together by the end of 3\textsuperscript{rd} week of gestation. Eventually they give rise to a neural tube close to the notochord’s anterior end. After formation of the neural tube, a set of cells become detached from the surface ectoderm at the tip of the neural fold giving rise to the neural crest. This eventually forms the sensory ganglia of cranial and spinal nerves. The neural tube is completely formed at around the 10.5\textsuperscript{th} or 11\textsuperscript{th} day of gestation (GD) in rats and 26\textsuperscript{th}, 27\textsuperscript{th} or 28\textsuperscript{th} day of gestation in humans. Closure of the neuropore occurs at 10.5\textsuperscript{th} day and 24\textsuperscript{th}-26\textsuperscript{th} day of GD in rats and humans, respectively. Closure of the posterior neuropore occurs later at 11.3\textsuperscript{rd} day and 25\textsuperscript{th}-28\textsuperscript{th} day of GD in rats and humans respectively. Serious problems of the brain and spinal cord could be encountered if neural development at this early stage is disturbed (Herschkowitz et al., 1997; Rice and Barone, 2000).

During the initial periods of the first month of gestation in humans and the second week of gestation in rodents, certain regions of CNS start forming through neurogenesis and cell migration in the hindbrain, midbrain and forebrain. This is followed by a cascade of developmental events that include proliferation, migration, differentiation, synaptogenesis, apoptosis and myelination, as shown in Figures 1 (Rice and Barone, 2000).
Figure 1: Rats nervous system development timeline in comparison to timing of fertilization, organogenesis, and histogenesis.

Modified from (Rice and Barone, 2000)
Alcohol consumption during pregnancy

Prenatal alcohol exposure could happen in two different ways; drinking while unaware of the pregnancy, or through an addiction to alcohol making it difficult to stop drinking even after being aware of pregnancy. It has been established that around 3.3% of pregnant women continue to consume alcohol chronically (defined as 7 drinks/week) or binge drink (when 5 drinks are consumed per occasion) (Burd et al., 2004). Alcohol consumption in pregnancy causes many spectrums of teratogenic effects and lifetime implications for a child. These disabilities are grouped under the general term “fetal alcohol spectrum disorder (FASD)”, which classifies the overall alcohol-induced effects observed in a child prenatally exposed to alcohol.

Ethanol metabolism

Researchers have shown three pathways for alcohol metabolism in humans. Enzymes required for these pathways include catalase, microsomal ethanol oxidation system (MEOS), and alcohol dehydrogenase (ADH). Free radicals can be generated in these metabolic pathways which influence the antioxidant functioning of the system. For instance, free radicals are produced in the classical pathway during which ADH converts ethanol into acetaldehyde. In this case, changes in the concentration of NADH and NADH/NAD+ redox ratios are recorded simultaneously (Cederbaum, 1991; Das and Vasudevan, 2007; Mantle and Preedy, 1999).

During moderate alcohol consumption, most of the consumed ethanol is subjected to metabolism in the liver by ADH.

\[ \text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ \rightarrow \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+ \]

In the above mentioned reaction, a hydride ion from ethanol is transferred to NAD+ (Cunningham and Bailey, 2001).
Oxidation of ethanol may also involve catalytic activity of cytochrome P450 isoenzymes. Hence it can be stated that the microsomal electron transport system has a role in ethanol oxidation. Isoforms cytochrome P450 2E1, 1A2 and 3A4 are the enzymes included in this family (Salmela et al., 1998) and these enzymes demonstrate varying potentials for ethanol oxidation. Basically, the reaction mentioned below is catalyzed by these enzymes.

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{CH}_3\text{CHO} + \text{NADP}^+ + 2\text{H}_2\text{O}
\]

Chronic consumption of ethanol induces the cytochrome P450 2E1 isoform. For that reason, alcohol consumption needs to be minimized since the quantity consumed may affect the mechanism induced. Besides this mitochondrial reaction, the cytochrome P450 2E1 isoenzyme may also contribute to generation of ROS in the individual consuming alcohol since its ability to produce large quantities of H$_2$O$_2$ is proven (Nordblom et al., 1977). Increased concentration of this enzyme in the liver of the alcoholic individual is associated with higher production of hydroxyl radicals (Klein et al., 1983).

As shown in the reaction given below, peroxisomal activity also plays an important role in ethanol oxidation taking place in liver.

\[
\text{RCH}_2\text{CH}_2\text{COSCoA} + \text{O}_2 \xrightarrow{\text{(AcylCoA oxidase)}} \text{RCH} = \text{CHCOSCoA} + \text{H}_2\text{O}_2
\]

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O}_2 \xrightarrow{\text{(Catalase)}} \text{CH}_3\text{CHO} + 2\text{H}_2\text{O}
\]

Individuals consuming large quantities of ethanol demonstrate deposits of fatty acids in the liver and accordingly this mechanism is likely to be more obvious in such cases (Rubin et al., 1972). Aldehyde dehydrogenases (ALDH) in the liver are responsible for oxidizing acetaldehyde, which is generated through the oxidation of ethanol.

\[
\text{CH}_3\text{CHO} + \text{NAD}^+ \rightarrow \text{CH}_3\text{COOH} + \text{NADH} + \text{H}^+
\]
ALDH present in the mitochondria makes a significant contribution in maintaining acetaldehyde levels at a desirable level. Acetyl CoA synthase acts on acetate to form acetyl CoA (Cunningham and Bailey, 2001).

The NADH / NAD\(^+\) ratio in the liver rises substantially as a consequence of ethanol oxidation by ADH and the ensuing acetaldehyde oxidation. This change is seen in the mitochondria as well as the cytoplasm as indicated by the lactate/pyruvate and \(\beta\)-hydroxybutyrate/acetoacetate ratios. Mitochondrial forms of the enzyme ALDH produce most of the NADH inside mitochondria. The malate-aspartate shuttle is responsible for transporting the reducing equivalents of NADH from the cytoplasm into the mitochondria. In the case of aerobic systems, the mitochondrial electron transport system quickly reoxidizes this added NADH. Therefore, availability of NADH in the mitochondria is enhanced by the oxidation of ethanol.

The ADH system is responsible for the degradation of alcohol in the liver. Apart from alcohol, the ADH gene family also produces enzymes that are responsible for the degradation of various other substrates such as ethanol, Vitamin A, aliphatic alcohols, hydroxysteroids and products of lipid peroxidation (or fat degradation). Humans contain around five different classes of ADH (i.e. Class I to V), and other species of animals such as rats and chickens include in addition to these classes, Class VI and VII (Duester et al., 1999).

These ADH enzymes break down or oxidize alcohol at different rates. The highest in terms of efficacy is the ADH1 (isoenzyme of class 1 ADH). ADH4 has ten times lesser efficacy and ADH3 becomes inactive in the presence of alcohol. Experimental trials performed on mice who lack the ADH 1 gene has demonstrated that ADH1 has a major role (75% to 90%) in metabolizing alcohol (To et al., 1999). The ALDH enzyme system, which is further broken down into three classes, is involved in metabolizing the by-product acetaldehyde into acetate. ALDH2 has the highest efficacy in oxidizing this by-product (Duester et al., 1999).
Fetal alcohol spectrum disorder (FASD)

FASD can have various clinical appearances depending on the extremity of abnormalities. The Institute of Medicine presented four main FASD categories; FAS, partial FAS, alcohol-related neurodevelopmental disorders and alcohol-related birth defects (ARBDs) (Abadir and Ickowicz, 2016). This disorder has specific identifying patterns including facial anomalies, growth deficiency, and central nervous system (CNS) dysfunction (O’Leary, 2004). Midface hypoplasia, short palpebral fissures, thin upper lip, epicanthal folds and cleft palate are the most commonly observed facial abnormalities. Growth deficiency, both prenatally that may cause a low birth weight in relation to gestational age, and postnatally, is a key characteristic of FAS (Abel and Hannigan, 1995a; O’Leary, 2004).

The child may be suffering from postnatal growth deficiency if the child’s height-to-weight ratio falls below the 10th percentile or is disproportionately low (Chudley et al., 2005). There are numerous dire effects that alcohol may produce on the development of CNS. These effects include brain abnormalities such as microcephaly, decreased intellectual ability, behavioural abnormalities, and impaired development of social, mental, and motor skills (Abel and Hannigan, 1995a; Chudley et al., 2005). Patients exhibiting the facial symptoms of FAS (i.e. short palpebral fissure length, smooth or flattened philtrum, thin upper lip) along with either growth restriction, CNS anomalies, or behavioural and cognitive abnormalities may indicate partial FAS (Chudley et al., 2005; O’Leary, 2004). Abnormalities of the CNS associated with FAS are also found in alcohol-related neurodevelopmental disorder, along with various physical and congenital abnormalities (Chudley et al., 2005). FAS may not be present in every child having prenatal exposure to alcohol; however, ARBD is present in them in one form or the other.
Alcohol effects on fetal development

Prenatal alcohol exposure interrupts fetal development directly and indirectly. There are many factors which can affect the extent of alcohol-induced damage in the fetus. The most important factors include: the level of alcohol consumed, pattern of consumption, duration of fetal exposure, specific stage of exposure during fetal development, and the mother’s nutritional status.

Direct effects on the fetus

Alcohol has the ability to freely flow through blood-brain barrier and the placenta, and therefore it can be easily diffused into any aqueous part of the body including lipid membranes and neurons (West and Blake, 2005). Microcephaly, which decreases the total brain weight up to 12%, may be caused by reduced DNA translation from reduced protein synthesis, and this reduced protein synthesis is due to alcohol exposure of the fetus (Miller, 1996; West and Blake, 2005). The brains of rats, mice, and humans have experienced impairment of developing neurons in the hippocampus when exposed to alcohol during prenatal and postnatal periods, which affects the memory processes, learning, and behaviour (Ikegami et al., 2003; Young et al., 2014). During developmental stages, even 1 or 2 days of alcohol exposure can cause neuronal death and irreparable damage to the brain and CNS. The inadequacy of FASD children in their sensory and motor capabilities is most probably due to the mass cell death and impaired neural development.

Indirect effects on the fetus

Alcohol affects the fetus in multiple ways and interrupts its development and growth through maternal hypoxia, oxidative stress, and altered metabolism. The morphological irregularities which develop in the fetus, which are also indicators of FASD, are caused by the altered cellular
functions during fetal development, and this alteration is caused by hypoxia (Bonthius and West, 1990; Pierce and West, 1986a). The fetus is sensitive during its development, and as a way of protection, the umbilical vessels vasoconstrict in response to alcohol exposure, as is the case with the placenta. Alcohol consumption interrupts the transport of oxygen to the maternal serum. In addition, vasoconstriction of the umbilical vessels greatly lowers the amount of oxygen reaching the fetus, affecting the neuron and neurotransmitter rich cerebellum and hippocampus (Pierce and West, 1986b). The CNS is damaged due to the high degree of glutamate and aspartate released by the neurotransmitters (Abel and Hannigan, 1995b). Reduced ATP production, impaired sodium-potassium adenosine triphosphatase (Na\(^+\)/K\(^+\) ATPase), increased lactate production, swelling of the mitochondria and inhibition of protein synthesis are dire consequences of hypoxia at the cellular scale. Fetal acidosis may be caused by growth retardation resulting from reduced ATP production and oxidative phosphorylation, affecting brain development and functions (Abel, 1995). Fetal growth is also interrupted due to lower transportation of important nutrients.

Cellular damage can be prevented by keeping a crucial balance between free radicals and antioxidants. Free radicals like \(O_2\) and \(H_2O_2\) are products of free radical production initiated by the consumption of alcohol. The greater the consumption of alcohol, the weaker the ability of internal oxidants to neutralize the free radicals produced, which causes lipid oxidation. There are various effects of lipid peroxidation which include alteration of membrane fluidity, membrane fatty acids, and phospholipid and glycolipid profiles and reduced activity of certain enzymes, which in turn cause changes in cell activity and function (Abel, 1995). As a consequence of alcohol consumption, neural crest cells become very vulnerable to oxidation as they do not have superoxide dismutase (SOD) (Davis et al., 1990). It is observed that the addition of vitamin E (an antioxidant) or SOD is employed for the attenuation of neural crest cell death which may be the primary cause of facial abnormalities shown by FAS patients. The neutralization of free radicals by cells and enzymes in the fetus is similar to the metabolism of alcohol by the placenta. But due
to the increased sensitivity of fetal cells to alcohol and decreased antioxidant levels, these compensating mechanisms do not measure up to the challenge.

Mechanism of alcohol induced brain damage

Alcohol causes brain damage by different mechanisms such as inducing oxidative stress and interrupting gene expressions. These mechanisms are explained further in the following sections.

Alcohol-induced oxidative stress

Owing to its solubility in lipids as well as water, ethanol is capable of diffusing readily into the stomach and esophagus by passing through the mucous membrane. Once absorbed, it is excreted both in urine and expired air. All ingested ethanol is subjected to oxidation and none is stored in the body (Wyatt et al., 1986). Metabolism of ethanol has a direct role in generation of ROS as well as development of an environment which favours oxidative stress including cytokine discharge, endotoxaemia and hypoxia (Sergent et al., 2001). Additionally, alcohol can aid generation of ROS through affecting the concentration of metals in the system.

The concentration of antioxidants which remove ROS from the cell is also negatively affected by alcohol. In other words, ethanol consumption is followed by the establishment of a marked imbalance between antioxidants and pro-oxidants that leads to oxidative damage of vital molecules like DNA, protein, fats and may result in cell injury (Sergent et al., 2001). According to Pemberton (2005), oxidative stress is the phenomenon which serves as the link between alcohol consumption, hepatic inflammation and fibrosis. Moreover, some research has reported that oxidative stress induced by ethanol plays a crucial role in triggering immune reactions against allo- and self-antigens in liver (Howell et al., 2002).
Oxidative damage in the brain

All aerobic organisms are subject to oxidative stress as mitochondria produce semi-reduced oxygen species, $\text{H}_2\text{O}_2$ and superoxide during respiration (Floyd et al., 1999). Although, several parameters can affect ROS production, about 2% of total oxygen consumed during respiration is considered to be accounted for by the amount of ROS produced. The ease of peroxidation of brain membranes supports the observed sensitivity of brain cells to oxidative damage (Wu et al., 2014). The rationale behind this is illustrated in a simplified form in figure 2. There is insufficient antioxidant defense in the brain, and therefore the brain consumes an inordinate proportion (20%) of total oxygen utilization for its relatively low weight (2%) owing to the presence of easily peroxidizable fatty acids. Catalase activity is low in the brain, about 10% of that in the liver. Generally, there is a high level of ascorbate in the brain, and in particular the brain contains higher levels of iron (Fe) in certain regions. So, ascorbate/iron is suspected to be an abnormal potent pro-oxidant for brain membranes, if disruption of tissue organization occurs.

When measured rigorously, the production of $\text{H}_2\text{O}_2$ from isolated brain mitochondria, about 2% of total oxygen consumption was shown to be accounted by $\text{H}_2\text{O}_2$ on the provision of reducing equivalents by NADH. Many oxidative processes as well as mixed function oxidase are other sources of ROS in addition to mitochondria. Production of $\text{H}_2\text{O}_2$ from oxidative deamination of catecholamines is of special importance for the brain (Peng et al., 2005).
Figure 2: Summary of brain poised to undergo oxidative damage. Based on the results from following studies: (Bansal et al., 2010; Van der Heyden et al., 2011; Muriach et al., 2014)

Alcohol induced gene expression

Until recently, advances in our knowledge of genes associated with alcohol consumption have been slow to materialize. However, there are some studies showed specific genes could be altered by alcohol consumption (Rietschel and Treutlein, 2013).

The differentiation of radial glial cells and their transformation into astrocytes are associated with alteration in gene expression, organization, and content of glial fibrillary acidic protein (GFAP) in astroglial cells. Ethanol can change GFAP gene expression and affect astrocytes and many astrocyte-mediated developmental events in the CNS, such as boundary formation during neural morphogenesis, central compartmentalization, neural proliferation and migration, axon outgrowth and guidance, availability of trophic support molecules, and myelination (Valles et al., 1997).

A study by Zhou (2011) identified a set of genes susceptible to alcohol exposure and genes that were linked with neural tube defects during early neurulation. Alcohol caused teratogenesis in the brain, and some of the embryos showed cranial neural tube defects. Based on microarray...
analysis, a collective reduction in the expression of neural specification genes [neurogenin, SOX5, BHLHE22] and neural growth factor genes [IGF1, EFEMP1, KLF10 (TIEG), EDIL3] have been reported (Zhou et al., 2011).

The Nmda-type glutamate receptor (Nmdar) is a critical player of synaptic plasticity, learning and memory (Malenka and Nicoll, 1999). Based on the literature, Nmdar is one of the major targets of ethanol in the brain and this receptor has been implicated in ethanol associated phenotypes (Krystal et al., 2003). Mandal et al (2015) reported that extreme ethanol exposure reduces Nmdar activity. This study also reported that nuclear factor I A (NFIA) gene expression was reduced in the hippocampus region. The alteration of gene expression in the brain caused by alcohol exposure elucidates disruption in normal molecular functions and may be responsible for the etiology of FASD (Mandal et al., 2015).

Maternal nutrition and alcohol consumption

Nutrition plays an important role in assuring a normal pregnancy. It has been established that alcohol has a significant correlation with nutrient intake during pregnancy on fetal development. The mechanism of action of alcohol is via blocking the supply of adequate nutrition to the placenta, and further if the mother has an inadequate diet it will lead to amplifying the effects of alcohol (Dreosti, 1993).

The relationship between nutrition and fetal alcohol toxicity was brought into light in 2007 by Shankar and colleagues when around 63 % of dams (pregnant female rats) demonstrated whole-litter resumptions (did not carry their litter to term) while constantly being given alcohol (13 g/kg/d) and also being given inadequate nutrition (given around 70 % of normal caloric intake, 160 Kcal/kg/d), while on the other hand the rest of the dams were given sufficient nutrition and the exact same dose of alcohol (Shankar et al., 2007).
Nutrition and alcohol interactions

Several possible mechanisms underlying alcohol–nutrition interactions have been explored. The pregnant rats that were not given adequate nutrition could not metabolize alcohol as effectively as compared to the pregnant rats that were given proper nutrition. This might be one possible reason responsible for exposing the fetus to risks evidenced by elevated levels of alcohol in the amniotic fluid and blood. The level of ADH1 in liver was also found to be lower in the malnourished pregnant ethanol-fed dams (Shankar et al., 2007). The exact role of ADH1 on nutrition is still not clear but data at hand has indicated that inadequate nutrition can cause significant changes in the ADH1 messenger RNA (mRNA). Other hormones such as growth hormone, thyroid hormone, and androgens that can affect ADH mRNA levels might also be modulated by nutrition. ADH transcription is also regulated by a number of proteins which have a direct role in transferring genetic information from the DNA to RNA (i.e., transcription factors), including CCAAT/enhancer–binding protein (C/EBP-β), sterol regulatory element–binding protein (SREBP-1) (He et al. 2002, 2004), and signal transducer and activator of transcription 5B (STAT5b). C/EBPs and SREBPs are known transcription factors that also have a significant part in energy metabolism (He et al., 2002, 2004).

Docosahexaenoic and alcohol

Docosahexaenoic (DHA) plays an essential role in central nervous system development. It is a precursor of a potent neurotrophic factor (neuroprotection D1), which enhances cell survival in brain tissues and protects the brain against injury induced oxidative stress. Therefore, DHA is recognized as a critical nutrient for infant development.
Dietary fatty acid function in the brain development

Several hypotheses have been proposed to explain the role of DHA in the brain, which in general can be divided into properties conferred by lipid-bound DHA in the membrane bilayer and those related to unesterified DHA. Hydrophobic member core related properties of the membrane determine its functions, which include uninterrupted member protein interaction and great flexibility; these properties influence lipid raft formation, neurotransmission, and transduction (Grossfield et al., 2006). Gene expression regulation and activities of the ion channel are functions performed by unesterified DHA, which when metabolized yields neuroprotective metabolites in the brain (Bazan, 2016; Vreugdenhil et al., 1996). Research has also presented DHA as an integral part in neurogenesis, phospholipid synthesis, and turnover (Kawakita et al., 2006; Salem et al., 2001).

It was observed that the level of DHA in the brain was low during the neonatal period of mice, and it caused greater levels of anxiety and fear in later stages when the mutation in their fatty acid-binding proteins (FABP) gene was null; this proved that the development of such characteristics in early stages is dependent on DHA (Owada et al., 2006). Belonging to larger category of small cytosolic proteins, FABP are a multi-gene group which performs various functions as cytoplasmic fatty acid transporters, including acting as an integral part in fatty acid transfer to the membranes, regulating fatty acid effects on gene expression, and acting as metabolite synthesis precursors. There are two main categories of FABP. B-FABP is located in ventricular germinal cells and glial cells in the embryonic brain, and in developing and adult brains it is present in the astrocytes. The other type is heart (H)-FABP, which is also found in adult brains (Veerkamp and Zimmerman, 2001). The B-FABP expression is integral to neural migration and early neurogenesis, as its expression occurs during development, and is very similar to that of early neural differentiation. When provided with nutrition lacking n-3 fatty acids during development, rodents showed inadequate spatial reasoning and memory (Innis,
There was no exhibition of inadequate spatial reasoning or memory in B-FABP mice which signifies the role of DHA binding proteins in DHA delivery on a cellular level at later stages of development, and may determine these characteristics.

Peroxisome proliferator-activated receptors (PPAR), a group of nuclear transcription factors which regulate the transcription of target genes through retinoid X receptor (RXR) heterodimerization and specific binding with certain parts of DNA have n-3 and n-6 fatty acids as ligands. The expression of PPARγ varies in different developmental stages of mice brain, with it having a high expression in the embryonic brain and neural stem cells and a relatively decreased expression in the adult brain and neural stem cells. Due to its involvement in stem cell proliferation regulation, it also has a significant role in the regulation of early stage brain development (Wada et al., 2006). Research has shown that ligands for the brain RXR include DHA and arachidonic acid (ARA, 20:4(n-6)) (Lengqvist et al., 2004).

The combination of RXR and retinoic acid receptor (RAR) is an integral part of various developmental procedures which include activity-dependent plasticity, neurogenesis during embryogenesis, and morphological differentiation of catecholaminergic neurons. Their part in furthering the understanding of DHA significance in the brain functioning of adults is important due to their high degree of expression in the hippocampus (Rioux and Arnold, 2005). Genes which control synaptic plasticity, cytoskeleton and membrane assembly, and signal transduction and ion channel formation are the downstream targets of RAR-RXR signalling and are dependent on n-3 fatty acid for their expression; inadequate provision affects all the aforementioned processes (Lane and Bailey, 2005). The brain may be affected in terms of its functions if the gene expression is altered during early stages and may cause changes in molecular and morphological development.

Oxidative stress-induced induction of proinflammatory genes and apoptosis in the retina and brain require DHA as it plays an integral role in the inhibition of these processes. Neuroprotectin
D1, which is a strong inhibitor of oxidative stress induced apoptosis and cyclooxygenase 2, is produced through the unesterified DHA released by phospholipase A2, which yields docosoids upon further metabolism. Due to its free radical scavenging characteristics, DHA also plays an important role in the protection of lipids and proteins against peroxidative damage in developing and developed brains (Cao et al., 2004).

Alcohol effects on docosahexaenoic

The DHA condition of a developing fetus depends greatly on the consumption of alcohol by the mother during the course of the pregnancy. In addition to causing a lack of fatty acid rich foods in the mother’s diet, alcohol consumption also lowers the DHA levels in the body causing a decrease in the DHA transferred to the developing fetus (Haggarty et al., 2002). The hippocampal growth, development, and life of neuronal cells is affected and interrupted greatly by the apoptosis and neuron cell death caused by increased alcohol consumption (Kim, 2008). An experiment that compared the hippocampal cultures of rats to test for apoptotic cells showed that ethanol exposed samples had a higher rate of apoptotic death due to decreased DHA-dependent phosphatidylserine accumulation, which helps in protection against apoptosis (Akbar et al., 2006; Rogers et al., 2013).
CHAPTER II: EFFECTS OF DOCOSAHEXAENOIC ACID SUPPLEMENTATION ON GLOBAL GENE EXPRESSION IN FETAL BRAIN IN RATS EXPOSED TO ETHANOL PRENATALLY

Introduction

Fetuses could be exposed to prenatal alcohol by a woman who consumes alcohol while not knowing that she is already pregnant, or a woman who is addicted to alcohol so that she could not stop drinking even after being aware of pregnancy. Attempts to deal with these problems through awareness and counselling of potential mothers using alcohol have met with limited success and the end result is an increase in incidence of fetal alcohol spectrum disorder (FASD).

FASD constitutes multiple effects that include physical, brain and central nervous system disabilities. Alcohol exposure affects the development of the brain during critical periods of differentiation and growth in FASD fetus. The underlying molecular mechanisms and processes of the teratogenic effects of alcohol exposure remain poorly understood. Metabolism of ethanol has a direct role in generation of reactive oxidative stress (ROS) as well as development of an environment such with cytokine discharge, endotoxaemia and hypoxia that favours oxidative stress. Prenatal alcohol exposure induced changes in gene expression involved in signal transduction and neurological system processes, such as nuclear factor one alpha (NFIA), special AT-rich sequence binding protein 2 (SAB2) and transcription factor AP-2 beta (TCFAP2B), may cause changes in the brain and development of FASD (Mandal et al., 2015; Pandey et al., 2008).

In overall this study tries to understand the global gene profile changes in fetal brain with prenatal alcohol exposure.

Also it has been observed that the neuronal tissue of brain is found to have high concentrations of n-3 docosahexaenoic acid (DHA), as compared to other organs of the body. During the last trimester, the content of DHA in the fetus increases significantly and DHA gets rapidly
incorporated into phosphatidylserine synthesis and storage in the hippocampus (Farooqui et al., 2000). The gray matter (where brain cell bodies are found) of the brain is especially rich in DHA. Because this DHA accumulation into the CNS occurs over a specific and limited period of development. Sufficient maternal dietary intake is critical in storage of DHA to transfer it to the fetus (Guesnet and Alessandri, 2011). Phosphatidylserine is the major acidic phospholipid class in eukaryotic biomembranes and is especially enriched in neuronal membranes (Kim et al., 2010). Nutrient deficiency lowers the rate of phosphatidylserine synthesis, which in turn leads to decreased neuronal development and survival, and ultimately results in poor cognitive CNS development. DHA plays an important role in normal brain development, yet there is limited information on the protective effects of DHA on the developing brain exposed to alcohol.

On the other hand, it has been shown providing DHA supplementation induced changes in gene expression involved in synaptic plasticity and learning in the brain, such as transthyretin, α-synuclein, and calmodulins (Kitajka et al., 2004). However, the effect of DHA supplementation on the global gene expression has not been studied yet.

Therefore, the objective of this study is to investigate the effects of DHA supplementation on global gene expressions in rat fetal brain prenatally exposed to alcohol. We hypothesized that the supplementation of DHA during pregnancy will normalize alcohol induced altered gene expressions.

Experimental design and methods

This study was approved by the University of Manitoba Animal Care Committee and are in agreement with the Canadian Council on Animal Care and Use of Experimental Animals (CCAC, 1993).
Animals and Diets

Pre-conception adaptation and mating

Sprague Dawley female rats (n=12) at the age of around 9-10 weeks’ old were used for this study. Randomly selected animals (n=8) were adapted to 50% ethanol slowly by increasing the dose of ethanol from 1/3 to 3/3 to reach the final expected dose (3g/kg BW twice a day) (Chappell et al. 2007) for 3 days. Then 2 females were mated with 1 male overnight within a cage. The day of finding a positive vaginal plug and/or sperm in vaginal swab defined as a gestation day 0 (GD0). During the time of adaptation and mating period animals were fed with the control diet (no-DHA) as described below.

Diets

The half of ethanol treated pregnant animals were randomly assigned to either control or DHA supplemented diet (1.4%, w/w fatty acids), and non-alcohol treated animals receive 80% dextrose (w/w) isocaloric to ethanol: thus, total 3 groups were i) no-alcohol + (Cont, n=5); ii) ethanol + no-DHA (EtOH, n=5); iii) ethanol + DHA (EtOH+DHA, n=5). The control and ethanol groups were gavaged twice a day with dextrose and ethanol, respectively, at a dosage 3g/kg BW (Chappell et al., 2007). Dextrose was used as a source matching the energy and it does not have an adverse effect on central nervous system and oxidative stress at this dose.

The experimental diets and fatty acid compositions are shown in Table 1. These were formulated isocalorically, semi-purified and nutritionally complete (Table 1) (Sutherland et al., 1997, 2000). The fatty acid compositions were measured in our laboratory. The total calorie dense was 4.29 Kcal/g diet composed of carbohydrate (50%), protein (17.5%) and fat (32.5%), reflecting pregnant mother and women diet in North America (Hui et al., 2014; Am Heart Assoc.
2014). Animals were accessed to diet and water, ad libitum. The amount of food and water consumed by each dams noted 3 days a week. The body weight of dams noted daily.

All the dams were sacrificed at GD 20 and their blood was collected by heart puncture. The uterine horn was removed and placed on ice. All the fetuses were weighed and the euthanized by decapitation with sharp scissors as soon as being removed from the uterus. The blood of fetus was collected from trunk and brain was removed and kept at -80 °C for further analysis.
Table 1: Experimental diet formulation.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Cont</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal diet (g/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>187.5</td>
<td>187.5</td>
</tr>
<tr>
<td>Corn starch</td>
<td>336.0</td>
<td>336.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Fat</td>
<td>155.5</td>
<td>155.5</td>
</tr>
<tr>
<td>Non-Nutritive Cellulose</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix (AIN93M)</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Vitamin mix (AIN93VX)</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Inositol</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Tert-butylhydroquinone</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Fat mix (g/100g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef tallow</td>
<td>68.0</td>
<td>65.9</td>
</tr>
<tr>
<td>Canola oil</td>
<td>4.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Corn oil</td>
<td>26.5</td>
<td>25.7</td>
</tr>
<tr>
<td>Flaxseed oil</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>ARASCO</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>DHASCO</td>
<td>-</td>
<td>4.7</td>
</tr>
<tr>
<td>Fatty acids (%, w/w Fat)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>C16:0</td>
<td>20.0</td>
<td>19.8</td>
</tr>
<tr>
<td>C18:0</td>
<td>10.6</td>
<td>10.3</td>
</tr>
<tr>
<td>C18:2(6)</td>
<td>17.5</td>
<td>17.0</td>
</tr>
<tr>
<td>C18:3(3)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>C20:4(6)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>C22:6(3)</td>
<td>-</td>
<td>1.4</td>
</tr>
<tr>
<td>18:2(6)/18:3(3) ratio</td>
<td>17.8</td>
<td>17.9</td>
</tr>
<tr>
<td>PUFA/SFA ratio</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

All the basal diet ingredients and also beef tallow purchased from Dyets, PA, USA. Canola oil, corn oil and flaxseed oil obtained from (Loblaw, ON, Canada, ACH, TN, USA and Omega, BC, Canada, respectively. ARASCO and DHASCO donated from DSM, CA, USA.
RNA isolation and microarray processing

Frozen brains (n=3 per group) were homogenized using a PowerGen 125 Homogenizer. Total RNA was extracted using TRIzol (Invitrogen, Ontario, province Canada), as per the manufacturer’s protocol. For 100mg tissue, 1ml TRIzol was added to precipitate the RNA. The extracted RNA was resuspended in 20μl RNAase free water and incubated for 10 min at 60 ºC. RNA yield and integrity in each sample was assessed using a NanoDrop (Thermo Scientific, Ontario, Canada) and an Agilent 2100 bioanalyzer (Agilent Technologies, CA. USA), respectively. Targets were prepared using WT PLUS Reagent Kit (Affymetrix, CA, USA). All sample RNA concentrations were adjusted to 250ng in order to synthesize first strand and second strand cDNA. The second strand cDNA transcribed to produce cRNA. Then, 15μg of cRNA was used to synthesize 2nd cycle single strand cDNA and for preparation of biotinylated targets. The biotinylated targets were hybridized on GeneChip® Rat Gene 2.0 ST Array (Affymetrix, CA, USA) using a hybridization oven (Affymetrix, CA, USA) at 45ºC for 16 hours. Following hybridization, staining and washing of chips was done as per the manufacturer’s protocol on the Fluidics Station 450 instrument (Affymetrix, CA, USA). Stained arrays were then scanned using a GeneChip™ 3000 laser confocal slide scanner (Affymetrix, CA, USA). Raw images were quantified with Affymetrix GeneChip Command Console Software (Affymetrix, CA, USA). Probe level data were background corrected, normalized and summarized using GC RMA algorithm under FlexArray software. Summarized data were annotated using the rat annotation file (RaGene-2_0-st-v1.na35.rn5.transcript.csv.zip) and differentially expressed genes were identified using t-test with Log₂ (fold change) cut off ≥2 and P-value ≤0.05. T- test data were compared based on 1) Alcohol effects: brain of alcoholic group rats’ vs brains of control group rats and 2) DHA effects: brain of DHA group rats’ vs brain of control group rats.
Microarray data validation by using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

A select set of genes were chosen from the microarray dataset and also based on previous studies for array validation (Table 3). For cDNA synthesis the same RNA samples were used as array hybridization. Based on the supplier’s protocol (Applied Biosystems, Canada), by using a high capacity reverse transcription kit, strand cDNA was synthesized. By using the National Centre for Biotechnology Information (NCBI), from the mRNA sequence of the target gene, pairs of primers for each gene were designed. Then by using a CFX Connect TM qRT-PCR Detection System (Life Science Research, Bio-Rad, Canada), quantitative real-time PCR was conducted. Reactions were in duplicate including the forward and reverse primers of target gene, nuclease free water, samples cDNA and SYBR Green as a detector. ΔΔCt method were generated for data analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as housekeeping gene to normalize the expression of the target genes.
<table>
<thead>
<tr>
<th>Name</th>
<th>Synonyms</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCDHB6</td>
<td>Protocadherin Beta 6</td>
<td>Involved in the establishment and maintenance of specific neuronal connections in the brain</td>
</tr>
<tr>
<td>PCDHB12</td>
<td>Protocadherin Beta 12</td>
<td>Their specific functions are unknown but they most likely play a critical role in the establishment and function of specific cell-cell neural connections</td>
</tr>
<tr>
<td>NFIA</td>
<td>Nuclear Factor One</td>
<td>Controls gliogenesis and cell proliferation</td>
</tr>
<tr>
<td>NEUROD1</td>
<td>Neuronal Differentiation One</td>
<td>Involved in oxidative stress. Contributes to the regulation of several cell differentiation pathways</td>
</tr>
<tr>
<td>GRIN1</td>
<td>Glutamate Ionotropic Receptor NMDA Type Subunit 1</td>
<td>Plays a key role in synaptic plasticity, synaptogenesis, excitotoxicity, memory acquisition and learning</td>
</tr>
<tr>
<td>WDR92</td>
<td>WD Repeat Domain 92</td>
<td>Seeks to act as a modulator of apoptosis</td>
</tr>
<tr>
<td>OLR101</td>
<td>olfactory receptor 101</td>
<td>Involved in inflammatory process</td>
</tr>
<tr>
<td>PPP1R14C</td>
<td>Protein Phosphatase 1 Regulatory Inhibitor Subunit 14C</td>
<td>Influences neuronal activity, protein synthesis, metabolism, muscle contraction, and cell division</td>
</tr>
<tr>
<td>TAF1D</td>
<td>TATA-Box Binding Protein Associated Factor, RNA Polymerase I Subunit D</td>
<td>Plays a role in RNA polymerase I transcription</td>
</tr>
<tr>
<td>CLDN12</td>
<td>Claudin 12</td>
<td>Plays a major role in tight junction-specific obliteration of the intercellular space</td>
</tr>
<tr>
<td>FGF1</td>
<td>Fibroblast Growth Factor 1</td>
<td>Plays an important role in the regulation of cell survival, cell division and cell migration</td>
</tr>
<tr>
<td>Gene name</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>TAF1D</td>
<td>TCACCGCATTTCTCATCTAGT</td>
<td>ACACAGTCAACAGAATCCACCT</td>
</tr>
<tr>
<td>PCDHB6</td>
<td>GACCGGAGATTCTGGGACAAC</td>
<td>AAACATGGGTGACCCTAACACT</td>
</tr>
<tr>
<td>OLR101</td>
<td>TATCCACATGTTTTGCGCTT</td>
<td>GCTCAAGCCAACACTACACCCA</td>
</tr>
<tr>
<td>NFIA</td>
<td>CCACCTGACCCCCCTCTCAAC</td>
<td>GCTGTTTGACCACGACGTTC</td>
</tr>
<tr>
<td>FGF1</td>
<td>CTTACCACAGCAGCAGGAATG</td>
<td>GTGCGTTCAAGACAGGTCAG</td>
</tr>
<tr>
<td>NEUROD1</td>
<td>TCAACCCCCCGGACTTTCTTG</td>
<td>GGGGACTGGTAGGAGTAGGG</td>
</tr>
<tr>
<td>CLDN12</td>
<td>AGGAAGCTGCGACTCATCAC</td>
<td>AAGTGGCCTGTACATCAGG</td>
</tr>
<tr>
<td>WDR92</td>
<td>GACCCGAGGCAAAAAAGAGGA</td>
<td>ATGTCCCCATTGTCGTAGCC</td>
</tr>
<tr>
<td>PPP1R14C</td>
<td>GTAAAAGCCCCCAGGCATTCT</td>
<td>CGCGTGTGTAGTATGGGTCA</td>
</tr>
<tr>
<td>GRIN1</td>
<td>GAAAAACCTCGACCAACTGTCC</td>
<td>TCAGCAGAGCCGTACACATT</td>
</tr>
<tr>
<td>PCDHB12</td>
<td>GGGGGAAATGTCAAGCAAGTTG</td>
<td>TGTGCTGTAAGGAATAGGGA</td>
</tr>
</tbody>
</table>
Western blot

Brains (n=5 per group) were washed with phosphate buffered saline (PBS) and incubated in lysis buffer, cold protein extraction solution (Sigma-Aldrich, Ontario, Canada) containing freshly added protease inhibitors (Sigma-Aldrich, Ontario, Canada). Protein concentrations were determined using Synergy H1 hybrid reader (FLUOstar Omega Instruments, Ontario, Canada) and protein samples were prepared at a concentration of 1mg/mL and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with anti-PCDHB6 (Biocompare, South San Francisco, USA), anti-NEUROD1 antibody (Sigma Aldrich, Ontario, Canada) and anti-NFIA antibody (Abcam, Ontario, Canada). After incubating with appropriate secondary antibodies, proteins were detected using chemiluminescence kit (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher Scientific, Ontario, Canada). Protein bands were scanned using a densitometer (GS-800, Bio-Rad Laboratories, Hercules, CA, USA). Band densities were quantified using the Quantity One 4.5.0 software (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as the loading control to normalize protein levels.

Statistical analysis

To see the effects of EtOH and DHA diet on the body weight and diet intake, one-way repeated measures analysis of variance (ANOVA) was used and Duncan's multiple comparison, using SAS statistical package (version 9.2; SAS Institute Inc., Toronto, ON). In Microarray, Probe level data were background corrected, normalized and summarized using GC RMA algorithm under FlexArray software. Summarized data were annotated using the rat annotation file (RaGene-2_0-st-v1.na35.rn5.transcript.csv.zip) and differentially expressed genes were identified using t-test with Log2(fold change) cut off ≥2 and P-value ≤0.05. To see the effect of ethanol or DHA diet on brain, t-test were used to compare the EtOH and DHA+EtOH groups
with Cont group. The qRT-PCR values were reported by customizing the fold changes of the expression of the target genes more than 1.5 and less than 0.5. To compare all 3 groups for qRT-PCR value and Western blot, one-way ANOVA and DUNCAN for multiple comparisons were used. Significance was defined as P<0.05. All values are expressed as mean ± S.D.

Results

Effects DHA supplementation on dams’ weight gain and diet intake with chronic alcohol consumption

The alcohol exposure to dams did not lead to abortion in this study. Effects of alcohol on dam’s weight and diet intake were measured (Table 5). Diet and ethanol did not significantly influence weekly weights among the groups (Figure 3). The total weight gains of dam at GD20 were 97.8g ± 9.47, 96.9g ± 10.88, and 126g ± 20.48, Cont, EtOH and EtOH+DHA, respectively. No significant differences were found in the daily diet intake in any of the groups (Table 5), thus the food efficiency (calculated body weight gain (g)/ diet intake (g)) was not different between the groups (Figure 4).

Effects of DHA supplementation on fetus weight with prenatal ethanol exposure

Effects of ethanol on fetal body weight and brain weight were measured (Table 6). Diet and ethanol did not significantly influence fetal body weight and brain weight (Figure 5). The average body weight of the fetus was 3.3 ± 0.1, 3.6 ± 0.31 and 3.2 ± 0.002, Cont, EtOH and EtOH+DHA, respectively. Brain weight of the fetus was 0.1652 ± 0.003, 0.1672 ± 0.008 and 0.1576 ± 0.002, Cont, EtOH and EtOH+DHA, respectively. The relative weight (% w/w) of brain to body weight was similar among the groups (Figure 5).
Effects of the DHA supplementation on the fetal brain’s global gene expression with prenatal alcohol exposure

Effects of alcohol on fetal brain global gene expression were measured by microarray (Figure 6). A total of 3935 number of genes were identified in fetal brain. EtOH alone or with DHA did not significantly influence the fetal global gene expressions at the level of $\log_2(FC) \geq \pm 2$ (Figure 6). Based on running t-test, OLR101 significantly (p<0.02) underexpressed by EtOH but PCDHB6 overexpressed, in comparison to Cont. By providing DHA to EtOH group also OLR101 significantly (p<0.02) underexpressed in comparison to Cont.

Validation of microarray data using qRT-PCR

A group of genes were selected from the microarray data to validate the results using qRT-PCR. A total of 11 genes (PCDH6, PCDHB12, WDR92, OLR101, GRIN1, NEUROD1, NFIA, TAF1D, CLDN12, PPP1R14C and FGF1) in the fetal brain were measured (Figure 7) were selected based on the fold change customized to FC>2 or FC<0.5. The results of most of the genes tested were consistent with the microarray data in terms of the fold changes. In EtOH group PCDHB6 significantly (P <0.05) overexpressed but the expression decreased by providing DHA. WDR92 significantly (P<0.005) underexpressed in the EtOH treated groups regardless of DHA treatment in comparison to the Cont. Also NFIA, NEUROD1, GRIN1 and WDR92 were also significantly (P<0.05) underexpressed in EtOH+DHA in comparison to Cont (Figure 7).

Protein Expression levels of NFIA and NEUROD1

Effects of alcohol on the protein expression levels of NFIA and NEUROD1 in fetal brain were measured by Western blot (Figure 8 and 9). Diet and ethanol did not significantly influence protein expression level of NFIA and NEUROD1 in fetal brain (Figure 8 and 9). The density of
NFIA/GAPDH were 117 ± 34, 127 ± 35 and 122 ± 29, Cont, EtOH and EtOH+DHA, respectively and the density of NEUROD1/GAPDH were 180 ± 11, 124 ± 22 and 126 ± 17, Cont, EtOH and EtOH+DHA, respectively.
Figure 3: Effects of DHA supplementation on dam’s weekly weight (g) with prenatal ethanol exposure. Data are means ± S.D., n=3–4 per group. Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.

Figure 4: Effects of ethanol exposure and DHA supplementation on dams food efficiency (body weight gain(g)/ diet intake (g)).
Data are means ± S.D., n=3–4 per group. Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.

Table 4: Effects of DHA supplementation on dams body weight, diet intake and food efficiency with prenatal ethanol exposure.

<table>
<thead>
<tr>
<th>Period (week)</th>
<th>Cont</th>
<th>EtOH</th>
<th>DHA+EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>273.3 ± 24.01</td>
<td>263.7 ± 19.55</td>
<td>259.3 ± 21.96</td>
</tr>
<tr>
<td>1</td>
<td>286 ± 21.49</td>
<td>281.7 ± 21.51</td>
<td>282.1 ± 23.03</td>
</tr>
<tr>
<td>2</td>
<td>313.2 ± 26.32</td>
<td>310.7 ± 22.67</td>
<td>320.6 ± 29.64</td>
</tr>
<tr>
<td>3</td>
<td>371.1 ± 33.48</td>
<td>360.6 ± 30.43</td>
<td>385.3 ± 42.44</td>
</tr>
<tr>
<td>Diet Intake (g/d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>19.27 ± 6.03</td>
<td>16.60 ± 2.60</td>
<td>25.20 ± 11.65</td>
</tr>
<tr>
<td>2</td>
<td>17.79 ± 4.68</td>
<td>16.32 ± 4.45</td>
<td>17.82 ± 4.94</td>
</tr>
<tr>
<td>3</td>
<td>18.40 ± 4.45</td>
<td>17.57 ± 3.61</td>
<td>21.85 ± 7.08</td>
</tr>
<tr>
<td>Food efficiency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.25 ± 0.15</td>
<td>0.28± 0.22</td>
<td>0.26 ± 0.17</td>
</tr>
<tr>
<td>2</td>
<td>0.24 ± 0.09</td>
<td>0.26 ± 0.16</td>
<td>0.30 ± 0.13</td>
</tr>
<tr>
<td>3</td>
<td>0.58 ± 0.11</td>
<td>0.53 ± 0.41</td>
<td>0.68 ± 0.19</td>
</tr>
</tbody>
</table>

Data are means ± S.D., n=3–4 per group. Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.

Food efficiency was calculated as body weight (g)/ diet intake (g).
Table 5: Effects of DHA supplementation on fetus body weight, brain weight and brain/body weight with prenatal ethanol exposure.

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>EtOH</th>
<th>DHA+EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>3.33 ± 0.1</td>
<td>3.59 ± 0.31</td>
<td>3.23 ± 0.002</td>
</tr>
<tr>
<td>Male (%)</td>
<td>38</td>
<td>52</td>
<td>40</td>
</tr>
<tr>
<td>Female (%)</td>
<td>62</td>
<td>48</td>
<td>60</td>
</tr>
<tr>
<td>Brain Weight (g)</td>
<td>0.1652 ± 0.003</td>
<td>0.1672 ± 0.008</td>
<td>0.1576 ± 0.002</td>
</tr>
<tr>
<td>Male (%)</td>
<td>37</td>
<td>51</td>
<td>41</td>
</tr>
<tr>
<td>Female (%)</td>
<td>63</td>
<td>49</td>
<td>59</td>
</tr>
<tr>
<td>Brain/Body Weight (%)</td>
<td>4.87 ± 0.2</td>
<td>4.67 ± 0.9</td>
<td>4.86 ± 1.0</td>
</tr>
<tr>
<td>Male (%)</td>
<td>36</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Female (%)</td>
<td>64</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>

Data are means ± SD, n=12–17 per group. Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.
Figure 5: Effects of DHA supplementation on fetus brain (g) relative to body weights (g) with prenatal ethanol exposure.

Data are means ± SD, n=6–17 per group. Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.
Figure 6: Effects of DHA supplementation on fetus brain global gene expression by microarray with prenatal ethanol exposure.

Data are Log$_2$(FC) $\geq 2$, n=3 per group. * P-Value <0.02. Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.
Figure 7: Effects of DHA supplementation on fetus brain gene expression by qRT-PCR (Microarray validation) with prenatal ethanol exposure.

Data are fold change ≥ 2 and fold change < 0.5, n=5. * P-Value < 0.05. ** P-Value < 0.005.

Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.
Figure 8: Effects of DHA supplementation on Nuclear Factor I A (NFIA) expression levels brain tissue with prenatal ethanol exposure.

Representative protein bands presented here have been adjusted for brightness universally across the image. However, band density was measured using the original scans without any changes. Data are means ± SD., n=5 per group. Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.
Figure 9: Effects of DHA supplementation on Neurogenic differentiation 1 (NEUROD1) expression levels brain tissue with prenatal ethanol exposure.

Representative protein bands presented here have been adjusted for brightness universally across the image. However, band density was measured using the original scans without any changes. Data are means ± SD., n=5 per group. Cont, Control; EtOH, Ethanol; DHA+EtOH, DHA diet + Ethanol.
Discussion

This study examined for the first time, the effects of DHA supplementation on global gene expression in rat fetal brains prenatally exposed to moderate level of alcohol.

Providing DHA supplementation did not affect fetal brain global gene expressions with prenatal ethanol exposure at the level of $\log_2^{(FC)} \geq \pm 2$. However, PCDBH2 genes were overexpressed in fetal brain with EtOH exposure prenatally, but OLR10 were underexpressed at the level of $\log_2^{(FC)} \geq \pm 0.7$. In further analysis, qRT-PCR agreed with microarray data in fold changes (FC<2 or FC>0.5). DHA supplementation significantly (p<0.05) decreased the increased expression of PCDHB6 with EtOH, to the levels in the Cont group. EtOH also decreased WDR92 expression regardless of a DHA provision in comparison to the Cont. EtOH+DHA significantly decreased the expression of NFIA, NEUROD1, GRIN1 and WDR92 in comparison to the Cont. Western blot revealed that none of the proteins expressed significantly different among the groups.

Ethanol dose

In the present study, ethanol was administered via gavage to dams, 3g/kg BW twice a day during the entire gestational period. Many studies have used similar dosage of ethanol and duration (Burdge, 1997; Lengqvist et al., 2004). Blood alcohol concentration in previous studies with similar level of alcohol consumption were approximately 300-400 mg/dl which is similar to moderate alcohol drinking in humans equals to 1 drink/day for women (Otero et al., 2012). In earlier studies, the same dose of chronic administration of alcohol showed significant interruptions in fetal brain development with deficits in spatial memory, alteration in behavior and motor function defects (Burdge, 1998; Ryan et al., 2008; Thomas et al., 2010). Increased glutathione peroxidase activity and production of TBARS was also reported by this model (Cano
et al., 2001). Dextrose provided to the control animals to be isocaloric, is known not interrupts any gene expression pathway (Pfefferbaum et al., 2015; Zahr et al., 2014).

Diet formulation

To see the effects of DHA in fetal brain exposed to ethanol prenatally, DHA was provided in the diet at the concentration of 1.4%, (w/w, total fatty acids). These animals were also provided energy dense nutritionally complete diet. The calorie density (4.29 Kcal/g diet) was reflecting a typical intake of Canadian pregnant woman, which is composed of protein (17.5% of total Kcal), carbohydrate (50 %) and fat (32.5 %) (Hui et al., 2014). The DHA concentration is a reachable amount by daily intake. Similar studies which supplemented the dams with less than this dosage did not show any changes in the dams’ weight gain during pregnancy (Bagley et al., 2013; Hadley et al., 2016).

Dams and fetal weights

In the current study contrary to the expectation, ethanol administration did not affect dams’ weekly weights, fetus and brain weights. The diet intake and weight gain were similar among the experimental groups. The average diet intake of ethanol group in the current study was 16.6 ± 2.6 g/day and control, 19.2 ± 6.0 g/day. Previous studies reported that alcoholic rats consume less food than the non-alcoholic rats (Ludeña et al., 1983; Xu et al., 2015), however the effects of alcohol consumption on diet intake depend on the dose, pattern and administration route. Studies showed that high dose of alcohol consumptions such as binge models interrupts food intake significantly more than the moderate levels of alcohol consumption (Nelson et al., 2016).

The importance of maternal nutrition on fetal alcohol toxicity has been clearly shown in a study by Shankar et.al (2007), in which a comparison made between pregnant rats given alcohol (13
g/kg/d) while being undernourished (fed 70 % of normal caloric intake, 160 Kcal/kg/d) vs adequate nutrition. Undernourished pregnant dams not only failed to carry their litters to term (63%), also had greater fetal toxicity, as indicated by reduced pup numbers, full-litter resorptions, and reduced birth weight. Our present diet provided approximately 252.7 Kcal/kg/d, 225.3 Kcal/kg/d, 209.1 Kcal/kg/d, in the 1st week, 2nd week and 3rd week, respectively, which is much higher than the above study. This might be the reason that my diet mitigated the effects of alcohol as Shankar et al (2007) state that the enriched diet might have increased the alcohol neutralizing enzymes such as ADH1 and helps body to degrade alcohol more effectively, thereby no affects in dam and fetal growth.

Ethanol and DHA impacts on global gene expressions in fetal brain

Since DHA alone or alcohol alone affects specific brain gene expressions, it was of interest to examine the global gene expression profile in fetal brain in our animal model. The microarray data analyzed indicated that the global gene expression in the fetal brain was not affected at the level of $\log_2^{\text{FC}} \geq \pm 2$ by alcohol exposure under the present research conditions. An earlier study by Mandal (2015) looked at the changes in gene expression in the fetal brain (forebrain and hippocampus) caused by maternal binge alcohol consumption. Pregnant C57BL/6J mice were treated intragastrically with distilled phosphate-buffered saline (PBS) or ethanol (2.9 g/kg/d) from GD 8–12. Microarray analysis of both the forebrain and hippocampus at GD 18 revealed that ethanol exposure altered 62 and 297 genes in forebrain and hippocampus, respectively. The diet in their study was laboratory chow. Although, the species, and ethanol treatment regime is different from my study, the null effect of alcohol exposure in my study, could be because of the nutrient dense diet provided in that may have mitigated the effects of alcohol. Other study also showed alcohol toxicity is a brain region – specific (Oscar-Berman and Marinkovic, 2003), hippocampus is most affected by alcohol. The present our study focused on a whole brain, which
may have also affected the results. Another possibility is a small change in a specific region is remarkable comparing to the whole brain. Maybe the change is still there but it is not significant by studying the whole brain.

The fold change cut off in the current experiment design was ± 2 as a generally accepted in the other studies. For validation check with qRT-PCR, the cut off point to ± 0.7 was selected to choose 11 genes: protocadherin beta 6 (PCDHB6), protocadherin beta 12 (PCDHB12), nuclear factor 1 alpha (NFIA), Neuronal Differentiation 1 (NEUROD1), WD Repeat Domain 92 (WDR92), Glutamate Ionotropic Receptor NMDA Type Subunit ONE (GRIN1), olfactory receptor 101 (OLR101), Protein Phosphatase 1 Regulatory Inhibitor Subunit 14C (PP1R14C), TATA-Box Binding Protein Associated Factor, RNA Polymerase I Subunit D (TAF1D), Claudin 12 (CLDN12), and Fibroblast Growth Factor one (FGF1). The selected genes are involved in neuronal connections, cell proliferation, oxidative stress, apoptosis, mediates neuronal functions, inflammatory process, neuronal activity, RNA polymerase, structural molecule activity, and cell survival, respectively (Table 2). Among these genes, NFIA, NEUROD1, PCDHB6 involved in cell proliferation, oxidative stress and neuronal connections were selected for western blot to study the protein expression in the brain.

Protocadherin Beta 6 (PCDHB6) is a potential calcium-dependent cell-adhesion protein. It is involved in the establishment and maintenance of specific neuronal connections in the brain (Ota et al., 2004; Vanhalst et al., 2001). Among these genes, PCDHB6 was overexpressed with ethanol but DHA supplementation decreased its expression to the level of control. Whether this changes in gene expressions leads to protein changes are not tested since PCDHB6 antibody was not available from any companies. The present study is the first to look at the effects of ethanol on PCDHB6 expression, but needs to be further confirmed.

The nuclear factor 1 (NFIA) family is a critical player for the early development of the brain. The transcription factor NFIA controls gliogenesis and cell proliferation (Dubois et al., 2010). It
has been shown that mice lacking NFIA family display delayed early hippocampal development, including neurogenesis (Harris et al., 2013). Another study evidenced by binge alcohol exposure, NFIA gene expression reduced in the hippocampus region (Mandal et al., 2015). In the current study ethanol did not affect NFIA gene expression and neither NFIA protein expression. This study took whole brain for analysis and the previous studies have been done specifically on hippocampal tissues. Maybe gene expression changes are limited to hippocampal regions and get normalized when whole brain samples are taken.

Neuronal Differentiation 1 (NEUROD1) contributes to the regulation of several cell differentiation pathways, like those that promote the formation of early retinal ganglion cells, inner ear sensory neurons, granule cells forming either the cerebellum or the dentate gyrus cell layer of the hippocampus, endocrine islet cells of the pancreas and entero-endocrine cells of the small intestine (Boraska et al., 2006; Bucan et al., 2009; Liu et al., 2006). Also it is required for dendrite morphogenesis and maintenance in the cerebellar cortex (Kavoura and Ioannidis, 2005). Previous studies presented that alcohol affects NEUROD1 expression in the hippocampal (Mandal et al., 2015; Roybon et al., 2009; Taffe et al., 2010). In the present study ethanol did not affect the gene expression of NEUROD1 nor the protein expression of it in the whole brain.

WD Repeat Domain 92 (WDR92) seems to act as a modulator of apoptosis via activation of caspase-3 (Saeki et al., 2006). The effect of alcohol on WDR92 has not been previously studied. WDR92 selected for qRT-PCR showed similar fold changes as shown microarray indicating that ethanol decreases this specific gene’s expression in the brain.

This is the first study to show the effects of DHA supplementation on global gene expression in rat fetal brains prenatally exposed to moderate level of alcohol. However, the effect of DHA was minor in the present experimental condition. It is speculated that the nutrient dense diet provided in our study may have reduced the effects of ethanol. Further studies with comparing nutritionally balance vs poor nutrition diet may provide more definitive results.
CHAPTER III: OVERALL SUMMARY

The present study indicates that, under the current experimental conditions, ethanol exposure during entire pregnancy has minor impacts on global gene expressions in the fetal brain. DHA supplementation also had no significant effect on fetal brain exposed to alcohol. The findings from this study indicate that a nutrient dense diet, similar to the one provided in this study, might mitigate harmful effects of prenatal ethanol exposure. Previous studies indicate that nutrition may be the key to improve not just fetal brain development, but general healthy fetal development. Future exploration of studying global gene expression with a low vs. high energy dense diet will help to better understand the brain’s global gene expression in prenatal ethanol exposure.

Strengths and Limitations

This is the first study to assess global gene expression in the fetal brain with prenatal alcohol exposure and DHA supplementation. The study was designed to investigate whether DHA supplementation during pregnancy affects global gene expressions in fetal brains exposed to ethanol prenatally in rats. The diet formulations for energy consumption reflected the current patterns of Canadian pregnant mothers’ intake as well as North American women intake. The amount of DHA supplemented in the current study is a holistic dietary model to the general population that is representative of the physiologic intake level. The alcohol consumption model in this study was chronic and it is representing moderate drinking pattern during pregnancy. Several limitations are also present within this study design. Firstly, by providing a semi purified nutritionally complete diet even for control rats, I might have nullified the effects of alcohol. This made it impossible to assess the global gene expression changes due to alcohol exposure. Secondly although I made sure the rats were not stressed by gavage, some rats were more
sensitive than the others. In this study I have not looked at stress markers. Additionally, the number of brains used for analysis of gene expression was low, indicating perhaps a larger number of tissues can be used to confirm the findings. Another potential limitation of this study is the ability to extrapolate these results to human studies. In humans, women may start drinking alcohol a long time before pregnancy, or a high dose of binging around pregnancy, but in the current study the alcohol exposure was limited to the pregnancy duration.

Recommendations for future research

In order to obtain a greater and conclusive understanding on the effects of alcohol exposure and DHA supplementation on the fetal brain, there are numerous facets that must be further explored. The first recommendation is to obtain a larger sample size and conduct a similar study in order to garner more robust data. To study the effects of a well-nourished diet, a study could be aimed at observing global gene expression with a low energy dense vs. high energy dense diet and/or nutritionally balanced vs. poorly balanced diet. DHA may have different effects during different stages of fetal and pup development. The next recommendation would be to expand this study to the pups and follow the progress through their developing stages. Studying the effects of alcohol during the different trimesters of pregnancy needs to be done as well. In this study the supplemented DHA amount was obtainable with a normal diet; future study with a higher amount of DHA supplementation should be conducted. Also consuming DHA with an antioxidant may show more protective effects in the body, and warrants further study.


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