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PERSPECTIVES ON ALCOHOL TOXICITY IN MEDAKA EMBRYOGENESIS AND POSSIBLE PROTECTION BY ASIAN GINSENG (PANAX GINSENG): HISTOLOGICAL AND BIOCHEMICAL ANALYSIS

A dissertation presented for the partial fulfillment of requirements for the degree of Doctor of Philosophy in the Department of Pharmacology

The University of Mississippi

Mona H. Haron

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ABSTRACT

Alcohol is recognized as a classic teratogen capable of inducing a wide range of developmental abnormalities. Alcohol consumption during pregnancy may produce permanent brain damage in the fetus and is associated with the development of a life-long behavioral, social, and cognitive disorder now known as fetal alcohol spectrum disorder (FASD). The most clinically recognizable form of FASD is fetal alcohol syndrome (FAS) which is characterized by specific features including facial dysmorphogenesis, mental dysfunction, growth retardation, and cardiovascular and limb defects. Due to ethical constraints, human studies of FASD are very limited. Therefore our current understanding of FASD is mainly based on several animal models (vertebrate and invertebrate). The fish embryo, especially zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), are long-established models for research in developmental biology and have been used to explore alcohol’s effect on neurogenesis, cardiogenesis, intracellular signaling, neurobehavioral aspects, and apoptosis. Exposing Japanese medaka embryos to alcohol during development showed phenotypic features and biochemical parameters that are comparable to FASD phenotypes observed in humans. Asian ginseng (*Panax ginseng*, PG) root extract has been shown to have anti alcoholic activities in a number of studies.

In this study we have used medaka embryos to induce FASD like neurological defects. We have found that exposing medaka embryos to alcohol at early embryonic stages (neurula stage) resulted in underdevelopment of the cerebellar Purkinje cell layer; both in number and in
size of the cells. We have also investigated the ability of PG to function as a preventive agent of embryonic alcohol toxicity. Our findings suggest that PG has a significant protective effect against alcohol-induced neurological deformities in medaka embryogenesis. Moreover in our study we have investigated developing FASD models in medaka embryos after chronically treating parent fish both male and female with alcohol. Our results indicated that chronic exposure of the parent fish to alcohol causes decreased fecundity of the female fish, but did not result in apparent FASD phenotypes in the offspring.
DEDICATION

This work is dedicated to my parents, dear husband Moustafa Elghory, and my children
ACKNOWLEDGMENTS

I would like to express my greatest gratitude to everyone who provided me with help and support during my study. First of all, I would like to thank my adviser, Dr. Asok Dasmahapatra, for accepting me into his lab and providing me the opportunity to work in this interesting area of research as well as giving me a lot of experience that will help me in my future research career. I am also grateful to my committee members: Dr. Kristine Willett, for being so supportive during lab meetings and seminars, and for allowing me to use different equipment in her laboratory this has been a great help for me to complete my research. Dr. John Matthews is always there for me and all the other graduate students helping us through both educational and personal setbacks, and Dr. Mahmoud El Sohly for his great academic and moral support.

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Activity dependent neuroprotective proteins (ADNP)
Activity dependent neurotropic factor (ADNF)
Alcohol dehydrogenase (adh)
Alcohol 300mM (A 300)
Alcohol-related neurodevelopmental disorder (ARND)
Alcohol-related birth defects (ARBD)
Aldehyde dehydrogenase (aldh)
Agouti viable yellow allele (Avy)
Ascorbic acid 25mM (S25)
Ascorbic acid 10mM (S10)
Asian ginseng, Panax ginseng (PG)
Blood alcohol concentration (BAC)
British Royal College of Obstetricians and Gynecologists (BRCOG)
Cytochrome P4502E1 enzyme (CYP2E1)
Central nervous system (CNS)
Crude extract (CE)
Cyclosporine A (CsA)
Endothelial nitric oxide synthase (eNOS)
Food and drug administration (FDA)
Fetal alcohol effect (FAE)
Fetal alcohol Spectrum Disorder (FASD)
Fetal alcohol syndrome (FAS)
Glutathione reductase (GR)
Glutathione-S-transferase (GST)
Golgi apparatus (GA)
Hatching efficiency (HE)
Hours post fertilization (hpf)
Hematoxylin and eosin staining (H&E)
High-density polyethylene (HDPE)
High-performance liquid chromatography (HPLC)
Hydrogen peroxide (H2O2)
Hydroxyl radical (‘OH)
Korean Ginseng (KRG)
Insulin receptor (IR)
Intelligence quotient (IQ)
L1 cell adhesion molecule (L1CAM)
MicroRNA (miRNA)
N-acetyl cysteine 25mM (N25)
N-acetyl cysteine 10mM (N10)
Neural cell adhesion molecules (NCAMs)
Neurocranium cartilage (NC)

Neuroligin gene (*nlgn*)

Nicotinamide adenine dinucleotide (NAD),

Nicotinamide adenine dinucleotide phosphate (NADP)

Nitric oxide (NO)

Partial fetal alcohol syndrome (PFAS)

Peroxyl radicals (ROO⁻)

Protopanaxatriol-enriched extract (TE)

Protopanaxadiol-enriched extract (DE)

Plant homology domain (PHD) finger protein 8 (PHF8)

Polysialic acid (PSA)

Quantitative reverse transcriptase real-time PCR (qPCR)

Reactive Oxygen Species (ROS)

Retinoic acid (RA)

Retinaldehyde dehydrogenase (RALDH)

Single nucleotide polymorphism (SNP)

Sonic hedgehog (shh)

Sialyl transferase enzyme (ST)

Superoxide anion radical (O₂⁻)

Trabecular Cartilage (TC)

Trichloroacetic acid (TCA)
CHAPTER 1

INTRODUCTION

1.1 Alcohol toxicity

1.1.1 Embryonic alcohol exposure as a cause of birth defects

Alcohol is being recognized as the leading preventable cause of birth defects and developmental disorders in the United States (Bailey and Sokol, 2008). Approximately 1 percent of live births are affected by fetal alcohol spectrum disorder, while FAS has an estimated prevalence range of 0.5 to 7.0 cases per 1,000 births in the United States (Sampson et al., 1997, May and Gossage 2001, and May et al., 2009). Other studies have suggested that the rates could be higher due to undiagnosed and/or unreported cases (May et al., 2009). In some selected populations such as in Native American communities, FAS incidence is as high as 1 percent of live births (Duimstra et al., 1993, and Burd and Moffatt, 1994). Prenatal exposure to alcohol can cause children to suffer from changes in brain structure and related serious cognitive deficits and behavioral problems as well as physical abnormalities. Deficits in learning and memory as well as in executive brain function both in children with fetal alcohol syndrome and in children with less severe FASD impairments have been identified in a number of neuropsychological studies. Problems in behavior, such as alcohol and drug use, hyperactivity, impulsivity, poor socialization and communication skills were exhibited with higher incidence in children prenatally exposed to
alcohol. Brain imaging studies of these children have identified structural changes in
different brain areas including cerebellum, basal ganglia, corpus callosum, and hippocampus that
may account for the cognitive deficits. It costs up to $4 billion per year in the United States alone
to deal with the consequences of prenatal alcohol exposure (Harwood, 2000). These alarming
public health and economic concerns are driving research to develop systematic identification,
effective interventions, and improved outcomes for individuals with FASD (Spadoni et al.,
2007).

1.1.2 Fetal Alcohol Spectrum Disorders (FASD)

The negative effects of alcohol consumption on pregnancy outcomes have been observed
throughout history. However the teratogenicity associated with prenatal alcohol exposure was
not recognized until later in the 20th century. P. Lemoine and his associates in France first
described the disease in 1968 after observing over 100 children of alcoholic parents. FASD
describes a range of neurobehavioral and physical abnormalities that appear after birth. Fetal
alcohol spectrum disorder in itself is not a clinical diagnosis; however, it is used to describe the
full range of disabilities that would result from prenatal alcohol exposure including FAS. The
diagnosis of FAS and other alcohol related birth defects is based on three criteria: (1) growth
deficiency (small head size and small overall height) (2) central nervous system disorders; and
(3) a distinctive pattern of abnormal facial features.

Fetal alcohol syndrome (FAS) is the earliest diagnosable, most severe form of FASD, with the facial dysmorphogensis, growth deficiency, and cardiovascular and neurological
abnormalities (Astley, 2004). Partial fetal alcohol syndrome (PFAS) patients have a confirmed history of prenatal alcohol exposure with central nervous system damage at the same level as FAS. However PFAS patients lack growth deficiency or the complete facial stigmata. These individuals have the same functional disabilities but "look" less like FAS (Astley, 2004 and Stratton et al., 1996). Alcohol-related neurodevelopmental disorder (ARND) focuses mainly on central nervous system damage, rather than growth deficiency or facial features. Even though the behavioral effects of ARND may not be unique to alcohol the use of the term ARND must be within the context of confirmed prenatal alcohol exposure (Streissguth, 1997). Alcohol-related birth defects (ARBD) consist of a list of congenital anomalies with no key features of FASD; however the defects are linked to maternal alcohol use during pregnancy (Stratton et al., 1996). Fetal alcohol effect (FAE) has been described by Smith (1981) as an "extremely important concept". It has been used to describe humans or animals with alcohol related teratogenic effects without obvious physical anomalies. It is also used to highlight the detrimental effects of brain damage, regardless of the growth or facial features.

1.1.3 FASD characteristics

The most serious characteristics of FASD are the invisible symptoms of neurological damage that result from prenatal exposure to alcohol. Prenatally alcohol exposed children show evidence of changes in brain structure and function as well as behavioral abnormalities that appear to result from an insult to the brain. Most of the research conducted on affected individuals has focused on either the brain structural changes or behavioral effects of alcohol exposure. Only recently studies have started to explore the link between the two areas
demonstrating that changes in brain structure could consequentially affect behavior. Results of neuropsychological studies analyzing alcohol’s teratogenic effects on behavior and of brain imaging studies analyzing alcohol's effects on brain structure highlight the existing connections between these two areas of research (Mattson and Riley, 1998 and Roebuck and colleagues 1998). Adverse effects were observed at as low as one glass/day in a dose–response relationship study between some of the growth parameters (up to 6 years of age) and average amount of alcohol daily consumed by pregnant women. One glass/day was a level of drinking approved by the British Royal College of Obstetricians and Gynecologists (BRCOG) Guideline in 1994. Moreover one glass/day was found to have threshold effect for the reading and spelling achievement subtests (Day et al. 1994, and Goldschmidt et al., 1996). Different animal studies have shown that low blood-alcohol levels could produce both brain damage and behavioral abnormalities associated with permanent decreases in the Purkinje and granule cell numbers in the cerebellum (Napper and West, 1995). The importance of timing and dosage in the etiology of FASD has been strongly suggested from several studies in different animal models (Becker et al., 1996). Drinking high concentrations of alcohol (5 U, or 40 g) once or twice a week, would have a greater risk on development than the same amount being consumed over a longer period of time (Jacobson et al., 1993, Jacobson and Jacobson, 1994, and Streissguth et al., 1994). Finally, it is important to know that threshold values are based upon averages of consumption; therefore it is not appropriate to assume that levels below a threshold value are necessarily ‘safe’, because of the individual variations in alcohol metabolism and pharmacokinetics (Eckardt et al., 1998). Furthermore some groups of women or fetuses may be more susceptible or sensitive to prenatal alcohol exposures than others. It was found that the risk for intellectual impairment increases
substantially in the offspring of women who are pregnant, drinking alcohol, and over the age of 30 years (Jacobson et al., 1996).

Growth deficiency features for prenatal alcohol toxicity:

- Poor growth while the baby is in the womb and after birth
- Decreased muscle tone and poor coordination
- Delayed development and significant functional problems in major areas: thinking, speech, movement, social skills, etc., (as expected for the baby's age)
- Heart defects such as ventricular septal defect or atrial septal defect
- Abnormal facial features distinctive of FAS:
  - Flat midface
  - Underdeveloped jaw
  - Thin upper lip, with possibility for cleft lip or cleft palate
  - Small, widely spaced eyes
  - Epicanthal folds
  - Short, upturned nose
  - Smooth, wide philtrum

1.1.4 Neuropsychological aspects of prenatal alcohol toxicity

Prenatal alcohol exposure is associated with a wide range of deficits in areas of cognitive functioning (e. g., general intellectual functioning, learning of new verbal information, and performance on visual-spatial tasks) and fine-and gross-motor function. Many neuropsychological studies have analyzed the cognitive impairment of children with histories of
prenatal alcohol exposure. Although these studies have focused on children diagnosed with FAS, several analyses have included children with FAE or PEA. Noteworthy, many of these studies showed strong similarities in cognitive function deficits between children with FAS and children with FAE/PEA.

Studies of overall cognitive ability in FAS children showed that although they can range from intellectually deficient (IQ scores less than 70) to average (IQ scores between 90 and 109) the typical average IQ scores in FAS children is in the low 70s (at the borderline range of functioning). Children with FAE or PEA, even though they tend to show deficits in their IQ scores, are not as severely affected as in the children with FAS (Streissguth et al., 1991 and Mattson et al., 1997). Furthermore, a broad range of cognitive functioning areas in children with FAS, FAE, or PEA has been evaluated, including language skills, visual-spatial functioning, fine-motor behavior, nonverbal learning, and academic performance. Prenatally alcohol-exposed children with or without FAS showed significant impairments in all neuropsychological areas. High levels of prenatal alcohol exposure were related to increased risk for cognitive deficits, which can occur in children both with and without a diagnosis of FAS (Mattson et al., 2000).

1.1.4.1 Learning and memory

Results from animal studies have indicated that learning and memory can be affected by prenatal alcohol exposure. Moreover studies of children with FAS have supported this observation. A study by Mattson et al., 1996b, investigating verbal learning and memory in children with FAS and in non-alcohol-exposed control children found that the FAS children exhibited some deficits in memorizing verbal information. These deficits seemed to result from
difficulties with the acquisition of the information rather than with the ability to remember the information. In another study (Mattson and Roebuck, 1998) the authors reported similar deficits in the acquisition of nonverbal information in prenatally alcohol-exposed children. These results suggest that learning deficits can occur in both verbal and nonverbal arenas and are more likely to cause significant impairment in diverse areas of functioning. Some other studies suggested that children with FAS could perform well when memory function is tested in a different way. For example in tests of implicit memory (not under conscious control), subjects may successfully perform implicit memory tests, using information from previous tasks without being aware that they have done so (Mattson and Riley, 1999). These findings suggest that even though children with FAS may have significant impairments in learning new information, their overall memory function may not be as much affected. Thus prenatal alcohol exposure may be affecting specific aspects of memory function more than the others.

1.1.4.2 Executive functioning

Executive functioning is a higher-level cognitive ability, such as solving problems or planning ahead. Children with a history of prenatal alcohol exposure have exhibited impairments on executive functioning tasks (Kodituwakku et al., 1995; Mattson et al., 1999). Furthermore in these studies the children's deficits in executive function were not related to their overall intellectual function. A recent study among adults with FAS or FAE, reported that the individuals’ deficits in executive functioning were greater than what would have been predicted if they were related to their overall IQ scores (Connor et al., 2000). Deficits in executive functioning have real-life implications. For example, people suffering from deficits in executive
functioning may act without first considering the consequences of their behavior, or they may have difficulties with activities that require problem solving or with planning a sequence of activities. This may explain why children with a history of prenatal alcohol exposure, even those with average IQ scores, may have difficulties succeeding in school.

1.1.4 Psychosocial deficits and problem behaviors

From studies involving parental reports and interviews of prenatally alcohol-exposed children, not only do they suffer from cognitive deficits but they also are at high risk for behavioral problems that would interfere with their lives at school and in other social environments. These children appear to be at high risk for alcohol and other drug abuse, maladaptive behaviors, and psychiatric disorders (Streissguth et al., 1996). They are also more likely than non-alcohol-exposed children to be hyperactive, disruptive, impulsive, or delinquent (Roebuck et al., 1999; Mattson and Riley, 2000). Adolescents and adults with FAS often exhibit poor socialization and communication skills and are less likely to be living independently. These problems occur in people prenatally exposed to alcohol whether or not they exhibit the criteria of FAS; moreover they occur to a greater extent than would be predicted by the person’s general intellectual functioning or demographic factors (Streissguth et al., 1991 and Thomas et al., 1998).

The neuropsychological and behavioral deficits depict real-life implications of the effects of prenatal alcohol exposure, and they capture the indirect manifestation of brain damage. These kinds of deficits also provide evidence of underlying changes in brain structure and/or function. Direct effects on brain development from prenatal alcohol exposure were noted in the early reports of FAS (Jones et al., 1973). Autopsy studies of the brains of affected children showed
widespread brain abnormalities.

1.1.5 Structural brain changes in FASD

Imaging studies have revealed differences between the brains of prenatally alcohol-exposed and non-exposed individuals. Along with the small head size, which is one of the diagnostic criteria for FAS, imaging studies showed a decrease in the over-all size of the brain of FAS children (Roebuck et al., 1998). To determine whether this size reduction results from diffuse alcohol effect on all brain areas or is limited to specific regions, specific structures in proportion to overall brain size have been assessed. The investigations have focused on certain brain areas, e.g. the cerebellum, basal ganglia, corpus callosum, and hippocampus.

1.1.5.1 Cerebellum

The cerebellum is involved in both motor and cognitive skills and is located at the base of the brain. Damage to the cerebellum has been implicated in learning deficits as well as in balance and coordination. Learning deficits have been shown by the neuropsychological studies to be the result of prenatal alcohol exposure. The overall volume of the cerebellum was found to be disproportionately reduced relative to overall brain size in people with FAS compared with control subjects (Archibald et al., 2001). These findings agree with previous reports of reduced cerebellar size in FAS and PEA children (Sowell et al., 1996). Moreover, studies conducted in both humans and animals suggest that a specific region of the cerebellum, the anterior portion of the cerebellar vermis, is particularly sensitive to prenatal alcohol exposure (Goodlett et al., 1990 and Sowell et al., 1996). Identification of specific alcohol-induced damage to the cerebellum
could be of particular importance in seeking a characteristic pattern of neurocognitive deficit related to FASD (Spadoni et al., 2007).

1.1.5.2 Basal Ganglia

The basal ganglia are a group of nuclei, including the caudate nucleus, putamen, and globus pallidus. They have a significant role in motor abilities and cognitive functions. The basal ganglia are affected by heavy prenatal alcohol exposure and are reduced in volume in children with FAS and PEA. Detailed investigation of the different basal ganglia nuclei found that the reduction in size is not uniform and that the caudate nucleus appears to be the most affected (Mattson et al., 1996a, and Archibald et al., 2001). Skills such as inhibition of inappropriate behavior, the ability to shift from one task to another and spatial memory are impaired in people with prenatal alcohol exposure. These skills have been related to the basal ganglia in other populations, such as patients with Huntington’s disease (Mattson et al., 1996a, Mattson and Riley, 1999, and Archibald et al., 2001). Consequentially, it is possible that the reductions in the caudate nucleus size in children with prenatal alcohol exposure accounts for some of the cognitive deficits observed. This hypothesis is of particular importance because the caudate nucleus has extensive neural connections to the frontal lobes of the brain, which are known to mediate higher cognitive and executive functions.

1.1.5.3 Corpus Callosum

The corpus callosum is a bundle of nerve fibers that connect the two hemispheres of the brain, allowing the left and right sides of the brain to communicate. Deficits in attention,
intellectual functioning, reading, learning, verbal memory, and executive and psychosocial functioning, all of which are impaired in prenatally alcohol-exposed people have been linked to corpus callosum abnormalities. Autopsy reports suggest that the corpus callosum is vulnerable to prenatal alcohol exposure. People with FAS exhibit abnormalities ranging from a thinning to complete absence or agenesis of the corpus callosum (Roebuck et al., 1998). Researchers found that specific regions of the corpus callosum were disproportionately reduced in size (Riley et al., 1995). Jeret and Serur, 1991, and Riley et al., 1995, mentioned that the rate of agenesis of the corpus callosum in people with FAS is higher than with any other developmental disorder.

Researchers analyzed in more detail the shape and location of the corpus callosum of FAS and PEA children as well as that of control children. They confirmed that the corpus callosum was reduced in size, specifically in the splenium, and that it was also significantly displaced in three-dimensional space. This corpus callosum displacement was highly related to the children’s performance on a verbal learning task, with the children with the greater displacement exhibiting more substantial performance impairments (Sowell et al. 2001).

1.1.5.4 Hippocampus

The hippocampus lies deep within the temporal lobe of the brain. The specific function of the hippocampus in aspects of memory is controversial. In adults with hippocampal damage the most obvious effect is the loss of anterograde amnesia (the ability to store new memories). Several studies on animals have suggested that this area is affected by prenatal alcohol exposure (Berman and Hannigan, 2000). In a study by Archibald et al., 2001, it was reported that the reduction in the volume of the hippocampus was proportionate to the reduction in overall brain
size, whereas other brain areas showed greater reductions in volume. Behavioral studies have supported the hippocampus being affected in children with prenatal alcohol exposure. These children have been reported to suffer from deficits in spatial memory and other memory functions associated with the hippocampus (Uecker and Nadel, 1996).

1.1.6 Proposed mechanism of teratogenic effects of alcohol

The influence of alcohol on embryonic or fetal development can be classified as (1) direct, by passing across the placenta and interacting with fetal cells, or (2) indirect, by causing biochemical or physiological changes in the mother which can be detrimental to the fetus. However, to explain the FASD mechanisms at the molecular level, a growing number of hypotheses have been proposed.

1.1.6.1 Oxidative stress and reactive oxygen species

Neurodegeneration observed in FASD which includes reduction of neurogenesis, inhibition of differentiation, disturbance of neuron migration, alteration of cell–cell interaction, and induction of apoptosis can at least, in part, be contributed to oxidative stress (Brocardo et al., 2009). Alcohol generates oxidative stress manifested as reactive oxygen species (ROS) when it is metabolized to acetaldehyde, and from acetaldehyde to acetate. The breakdown of alcohol occurs in two steps. First, alcohol is converted to acetaldehyde by three independent pathways catalyzed by (1) alcohol dehydrogenase (ADH), (2) cytochrome P450 2E1 (CYP2E1), and (3) catalase. Acetaldehyde is then converted to acetate by the mitochondrial form of aldehyde dehydrogenase 2 (ALDH2) (Li et al., 2012). Each of these reactions leads to the reduction of one molecule of
nicotinamide adenine dinucleotide NAD$^+$ to NADH (in the case of CYP2E1, it is NADP$^+$ to NADPH) (Magni et al., 2008). As a consequence, the ratio of NADH/NAD$^+$ is significantly increased. This alters the cellular redox state and initiates a number of adverse effects (Norberg et al., 2003). Most of the cellular sources of ROS are associated with the generation of a superoxide anion radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), which are the precursors of the highly reactive hydroxyl radical (·OH). Once formed, hydroxyl radicals interact with carbohydrates, proteins, lipids, and nucleic acids to form peroxyl radicals (ROO$^-$) as intermediates that can propagate damage to biomolecules to produce cell damage and death. At present, most of the cited evidence for the involvement of ROS in FASD is indirect. Only a few studies have analyzed the production of ROS in animals exposed to alcohol during the prenatal period (Heaton et al., 2002, Heaton et al., 2003, Kane et al., 2008, and Dong et al., 2010). In a study by Arteel et al., 1996, it was concluded that one large dose of alcohol can cause per central hypoxia in rat liver tissue and that Kupffer cells are involved. Millicovsky, et al., 1981, reported that there is increased incidence of cleft lip/palate in mice that have suffered from hypoxia during pregnancy. PHF8 is from the family of ferrous iron dependent oxygenases, it is active as a histone lysine demethylase. The catalytic activity of PHF8 depends on molecular oxygen and is disrupted by hypoxia (Loenarz et al, 2009). In humans, mutations of PHF8 that cluster in the catalytic domain of this enzyme cause cleft lip/palate, facial dysmorphism, and other congenital abnormalities with mild mental retardation (Koivisto et al., 2007). These abnormalities and deformities are similar to those seen in FASD. Maternal smoking, alcohol consumption, and hypertension treatment have been linked to maternal hypoxia (Shi et al., 2008, Hurst et al., 1998, Cohen-Kerem, and Koren, 2003, Chen et al., 2004, Dong et
al., 2008, and Parnell et al., 2010). These reports indirectly support the theory that alcohol induced hypoxia and resulting oxidative stress is one of the potential mechanisms of FASD. This study investigated this potential mechanism.

1.1.6.2 Interference with retinoid metabolism

Retinoic acid (RA) is a metabolite of vitamin A (retinol) and a morphogen, governing cell differentiation and embryonic patterning in early developmental stages (Maden et al., 1989, and Mark et al., 1999). RA, along with some other molecules, is responsible for proper development of the craniofacial region and skeleton in vertebrates. In experimental animals, RA deficiency produces phenotypes that strongly resemble FAS (Deltour et al., 1996, and Kot-Leibovich, and Fainsod, 2009). Retinol, an isoprenoid, is converted to RA in a two-step oxidative reaction. Retinol is oxidized by alcohol dehydrogenase (ADH) (class IV) producing the intermediate retinal (retinaldehyde). Then, retinaldehyde dehydrogenase (RALDH) catalyzes the conversion of retinaldehyde to retinoic acid RA (Connor, and Smit, 1987). Alcohol competitively inhibits both ADH and RALDH, resulting in impaired RA biosynthesis. This leads to developmental malformations that mimic FAS (Deltour et al., 1996, and Kot-Leibovich, and Fainsod, 2009). Some studies found that RA supplements are able to prevent developmental defects in alcohol treated embryos of zebrafish, frog, and mouse (Yelin et al., 2005, Johnson et al., 2007, and Marrs et al., 2010).

1.1.6.3 Cholesterol deficiency and sonic hedgehog signaling

Cholesterol is an end product of the mevalonate pathway (Edison, and Muenke, 2004)
and is required for normal development. Alcohol inhibits the mevalonate pathway and thus cholesterol biosynthesis (Cottalasso et al., 1998, and Li et al., 2007). In mice, the lack of availability of cholesterol results in a drastic decline in sonic hedgehog (shh) signal transduction (Edison, and Muenke, 2004). Moreover, chicken and mouse embryos exposed to alcohol displayed reduced shh signaling (Guizzetti and Costa, 2007). Therefore, alcohol-dependent inhibition of the cholesterol modification of shh produces morphologic defects, which are similar to FASD phenotypes. In zebrafish embryos, alcohol treatment causes a dose-dependent reduction in cholesterol content, decreased cholesterol modification of shh, and a loss of shh signal transduction resulting in cyclopia, craniofacial hypoplasia, and holoprosencephaly. Supplementation of cholesterol was able to rescue the zebrafish embryos from this phenotypic defect (Li et al., 2007).

1.1.6.4 Interference with neural cell adhesion molecules during CNS development

Neural cell adhesion molecules (NCAMs) play a significant role in neuron–glia interaction, synaptogenesis, neuronal migration, growth, and morphogenesis in embryos (Minana et al., 2000). NCAMs require posttranslational glycosylation for proper neuronal cell migration in utero in the form of addition of multiple polysialic acid (PSA) residues. Sialyl transferase (ST) mediates this process in the Golgi apparatus (GA). Prenatal alcohol exposure inhibits ST activity and leads to cytoplasmic retention of PSA-NCAMs. The resultant reduction in cell surface expression and functionality of PSA-NCAMs is believed to underlie the migration-related errors, heterotopias, and other morphological brain defects as observed in FAS (Minana et al., 2000). Another important cell adhesion molecule in the developing brain is L1 (L1-CAM), a member of
the immunoglobulin superfamily, which regulates cell–cell and cell–matrix interactions, neural migration, neurite outgrowth, and axon guidance and fasciculation (Fitzgerald et al., 2011). Alcohol could induce FASD by disrupting L1-CAM-mediated processes (Charness et al., 1994, and Ramanathan et al., 1996). Glial derived activity dependent neuroprotective proteins (ADNP) and activity dependent neurotropic factor (ADNF) were found to block alcohol inhibition of L1 adhesion and also to prevent alcohol teratogenesis in mouse embryos (Spong et al., 2001, and Wilkemeyer et al., 2003).

1.1.6.5 Interference in insulin signaling

Insulin signaling, which is mediated by the insulin receptor (IR), is required for cell viability, metabolism, synapse formation, and acetylcholine production. Alcohol exposure inhibits insulin signaling at the IR level and thus causes neurodevelopmental abnormalities as observed in FASD (De la Monte and Wands, 2010).

1.1.6.6 Disrupting epigenetic mechanisms

Epigenetics is the field of research that examines alterations in gene expression caused by mechanisms other than changes in DNA sequences (Laracy et al., 2012). A mechanism that could underlie the actions of alcohol on FASD is disruption of epigenetic mechanisms mediated by DNA methylation, histone modification, and noncoding RNAs (Bernstein et al., 2007). Studies suggest that DNA methylation, posttranslational histone modification, and noncoding RNAs are altered in animal models of FASD (Kim et al., 2006, Lee et al., 2007, PalBhadra et al., 2007, Haycock, 2009, Liu et al., 2009, Kaminen-Ahola et al., 2010, and Zhou et al., 2011). The
administration of alcohol to pregnant mice, from day 9 through day 11 of gestation inhibits fetal DNA methylation (Garro et al., 1991). Disturbance of miRNA functions may contribute to the alcohol-induced developmental deficiencies (Wang et al., 2008). Several miRNAs, such as miR10a, miR-10b, miR-9, miR-145, miR30a-3p, and miR-152, were up regulated in fetal mouse brains with prenatal alcohol exposure, whereas miR-200a, miR-496, miR-296, miR-30e-5p, miR-362, miR-339, miR-29c, and miR-154 were down regulated. Both miR10a and miR10b are embedded in the Hox cluster during evolution, and miR10a, miR10b, and Hox genes are expressed in early embryo development (Wang et al., 2009). Alcohol exposure decreased expression of miR-21, miR-335, miR-9, and miR-153 in cultured neurospheres from fetal mouse cerebral cortex, lead to cell cycle induction and stem cell maturation (Sathyan et al., 2009). More importantly, a probable link between alcohol consumption by men with increased demethylation of normally hypermethylated-imprinted regions in sperm DNA and FASD is being investigated (Ouko et al., 2009). During development, there are essentially three main stages of generalized global epigenetic remodeling which can be targeted by alcohol exposure: (1) during gametogenesis, when there is a wave of demethylation followed by sex specific genetic imprinting and generalized methylation; (2) during preimplantation, which is characterized by generalized DNA demethylation in the zygote (with the exception of imprinted loci); and (3) during gastrulation, when another wave of de novo methylation is observed (Reik et al., 2001, and Ramsay, 2010). Taken together these factors indicate a link between alcohol-induced behavioral and phenotypic changes in humans and the epigenetic modifications that may occur due to alcohol consumption. However, few studies have addressed whether or not these epigenetic changes can be transmitted to the offspring. It has been observed that offspring of
alcoholics have shown cognitive, physiological and psychological problems with increased risk for developing alcoholism. This led us to our third goal in this study. We have exposed the adult Japanese medaka, both male and female, to alcohol followed by investigation of the epigenetic changes that could be transmitted to the offspring. We assume that some epigenetic changes could occur due to chronic alcohol exposure and that these changes can be transmitted to the offspring leading to some of the neurobehavioral and morphological deformities shown in FASD.

1.1.6.7 Altering placental metabolism

Good placental function is crucial for fetal development, and many pathways have been discovered where maternal alcohol consumption affects placental function and fetal development. Alcohol induces acute placental vasoconstriction that affects placental transport of essential nutrients such as biotin, amino acids, and vitamins (vitamin B6) (Burd et al., 2007) and thus affects fetal growth. Microarray analysis of placental genes in rat has demonstrated that the expression of 22 genes related to CNS development, organ morphogenesis, immune response, skeletal, vascular, and cartilage development were altered significantly after moderate chronic alcohol consumption (Rosenberget al., 2010). Moreover, acetaldehyde can also be generated in the placenta where the expression of CYP2E1 protein is related to alcohol consumption and thus cellular injury (Rasheed et al., 1997).

1.1.7 Prevention of embryonic alcohol toxicity

Although a lot of progress has been made in understanding the mechanisms of alcohol
teratogensis, challenges remain ahead. Many pregnant women continue to consume alcohol, and many women in their childbearing age consume alcohol at dangerous levels placing their potential offspring at high risk for negative outcomes. With this being the situation, prevention and intervention strategies have become very high priority (Kobor, 2011).

Prevention of FASD, other than women abstaining from drinking alcohol during pregnancy, is not known. The Surgeon General, US Department of Health and Human Services, in 2005, urged women who are pregnant, planning to be pregnant or may be pregnant to abstain from alcohol use. It is the last part of this advisory targeted at “women who might become pregnant” that leads us to another target group for prevention and treatment. It is believed that 1/3 to 1/2 of pregnancies are unintended. It was found that about 37% of pregnancies in the United States were unintended at the time of conception (Marchetta et al., 2012). Unintended pregnancies include both pregnancies that are unwanted and those that are mistimed, meaning the woman said she wanted to become pregnant at some point, but not at the time she did. There are a lot of reasons why a woman becomes pregnant unintentionally and misuse of contraception methods is one of them. Moreover researchers found that only 40% of women in the childbearing period use contraception (Mosher et al., 2012). Rates of unintended pregnancy in the U.S. are generally highest in the South and Southwest, and in states with large urban populations. The highest unintended pregnancy rates in 2006 were found in Mississippi (69 per 1,000 women aged 15–44), California (66), Delaware (66), the District of Columbia (67), Hawaii (66) and Nevada (66) (Finer and Kost, 2011).

The Centers for Disease Control and Prevention (CDC) released some reports in the United States with data on unintended pregnancies and the use of alcohol during pregnancy for
the years of 2006-2010. The first report looked at alcohol use and binge drinking among women of childbearing age. The researchers found that approximately 7.6% (or 1 in 13) of pregnant women consume alcohol during pregnancy and that 1.4% of pregnant women binge drink. Surprisingly, non-pregnant women and pregnant women had similar patterns of binge drinking: about three times per month and approximately six drinks per occasion (Chavaz et al., 2011). Among pregnant women, the highest prevalence estimates of reported alcohol use were among those who were aged 35–44 years (14.3%); “white” (8.3%), college graduates (10.0%), or employed (9.6%).

Taken together, this leads us to our second goal of this study. Finding a treatment that would ameliorate or prevent fetal alcohol toxicity is important because of the possible high risk for fetal alcohol toxicity. Moreover the disabilities induced in FASD continue throughout life, which causes emotional and financial suffering for individuals, families, and the whole community.

1.1.8 Possible treatments from natural products in Fetal Alcohol Spectrum Disorder

Anti-alcoholic drugs such as disulfiram, naltrexone and acamprosate, even though approved by FDA, are pregnancy category C (adverse effects on the fetus in animal studies but no human trials) (Cheng et al., 2005; Kondo, 2006). Therefore these compounds cannot be used in FASD prevention studies. Topiramate, gabapentin, ondasentron, and baclofen are compounds currently under investigation for their anti-alcoholic effect. This led us to investigate herbal sources of anti-alcoholic effect substances. Alcohol can disrupt several systems especially brain and cardiovascular system with several mechanisms. In our search for a good source for a drug
to treat FASD we searched in herbs with proposed neurological and cardiovascular protective effects. Kim et al., 2013, has stated “Central nervous system (CNS) diseases are the most widely investigated diseases among all others in respect to the ginseng's therapeutic effects. These include Alzheimer's disease, Parkinson's disease, cerebral ischemia, depression, and many other neurological disorders including neurodevelopmental disorders”. Possible ginseng- or ginsenosides-mediated neuroprotective mechanisms mainly involve maintaining homeostasis, and anti-inflammatory, anti-oxidant, anti-apoptotic, and immune-stimulatory activities (Ik-Hyun Cho, 2012, and Hu et al., 2011). The root of Asian ginseng has been shown to induce a hypotensive effect through nitric oxide (NO) release from the endothelial cells. However, the main active component contributing to vascular endothelium relaxation remains uncertain. The hypothesis is that multiple components of ginseng extract may have complimentary effects providing better results than a single ginsenoside. A wide range of ginseng extracts (crude extract, CE; protopanaxatriol-enriched extract, TE; protopanaxadiol-enriched extract, DE) and individual ginsenosides (Rg1, Re and Rb1) were compared for the NO-releasing and endothelial NO synthase (eNOS) activating potency in human umbilical vein endothelial cells. TE had the highest potency in NO production, followed by CE, DE, and Rg1 (Ahn et al., 2013). Another study was performed to investigate whether ginseng extract has a protective effect via suppressing oxidative stress resulting from chronic cyclosporine in an experimental mouse model of cyclosporine A (CsA) nephropathy. The conclusion was that KRG has a protective effect in CsA-induced renal injury via reducing oxidative stress (Doh et al., 2013). Administration of Asian ginseng (PG) in diabetic rats showed significant decreases in serum glucose and tumor necrosis factor-α (TNF-α), implying that PG might prevent the pathogenesis
of diabetic complications caused by impaired glucose metabolism and oxidative stress (Quan et al., 2013). These findings show a potential therapeutic effect for ginseng on FASD through improving the placental fetal blood supply, improving insulin metabolism and the relief of oxidative stress.

Studies on the effect of PG on improving alcohol teratogenicity showed that alcohol-induced reduction of neonatal brain weight in rats was prevented by the administration of ginseng extract (Okamura et al., 1994). Lee et al., 2009, stated “black ginseng has a protective effect on alcohol-induced teratogenesis through the augmentation of antioxidative activity in mice embryos”. Therefore, our hypothesis is that a safe and effective anti-alcoholic compound can be isolated from Asian ginseng root extract (black ginseng), which can be used for the amelioration or prevention of some features of FASD. In this study we focused on the possible anti oxidative properties of Asian ginseng in the amelioration/prevention of FASD using medaka as our animal model.

1.2 Asian ginseng, *Panax ginseng* (PG)

1.2.1 Position of Asian ginseng among medicinal herbs:

Asian ginseng has been one of the best-selling botanicals in the USA for many years (Bluementhal, 2001). More than six million Americans regularly consume ginseng products (Smolinski and Pestka, 2003). Moreover, ginseng has shown no adverse effect in human pregnancy (Seely et al., 2008). It is one of the eleven species of slow-growing perennial plants that has fleshy roots. It belongs to the genus *Panax* of the family Araliaceae. Ginseng can only be found in the Northern Hemisphere, it needs cooler climates e.g. North America, and eastern
Asia (mostly Korea, northern China (Manchuria), and eastern Siberia). There are five main species of ginseng: American, Chinese, Korean, Japanese and Siberian (or Russian) and it is important to be able to distinguish between them. The commercially available product ‘ginseng’ usually refers to the dried root of *Panax ginseng*, commonly known as Korean or Asian ginseng. Preparations of *P. ginseng* include the steam-dried root that is called ‘red ginseng’, and the air-dried root that is called ‘white ginseng’. Fresh ginseng extract is also consumed, but is not generally the preparation available commercially (Seely et al., 2008). *Panax* species are known for their adaptogenic activity, mainly *P. ginseng* and *P. quinquefolius*.

Ginsenosides or panaxosides are the characteristic components of *Panax ginseng* (Boon and Smith 2004). Ginsenosides are a class of steroid glycosides, and triterpene saponins. All ginsenosides have a common four-ring hydrophobic, steroid-like structure with attached sugar moieties. The specific action of each ginsenoside might depend on the diversity of the sugar components, and the number and position of the sugar moieties (Chen et al., 2008).

1.2.2 Ginsenosides as the active compounds behind ginseng activity

Ginsenosides have been viewed as the active compounds behind the claims of the different activities of ginseng. Ginsenosides seem to affect multiple pathways; therefore their effects are complex and difficult to isolate (Attele et al., 1990). The ginsenoside content of ginseng varies with the species, the plant age, parts of the plant, preservation methods, season of harvest, and extraction method (Liberty and der Mardersian, 1978; Phillipson and Anderson, 1984). Ginsenosides are found in the root of the plant and they can be categorized into two groups. The Rb1 group (characterized by the protopanaxadiol presence: Rb1, Rb2, Rc and Rd)
and the Rg1 group (protopanaxatriol: Rg1, Re, Rf, and Rg2). Ginsenosides can be separated into these pure compounds by column chromatography. The root is a thick structure that resembles a human-like form, giving rise to the name in Chinese, jen shen, or man-root. Panax is derived from the Latin word Panacea; it refers to its wide use for many conditions. Ginseng has been used in Asian cultures for conditions such as fatigue, mental stress, blood sugar regulation, improving libido, and supporting longevity. Modern studies and current clinical applications for ginseng have focused on ginseng in cancer prevention, immuno-modulation, anti-oxidant, anti-inflammatory, anti-hyperglycemic, anti-apoptotic activities, potentiation of nerve growth factor, and it may have a neuroprotective effect through nicotinic activity (Sotaniemi et al., 1995, Scaglione et al., 1996, and Hong et al., 2002).

1.3 Fish model in FASD

Limitations exist in using humans as subjects for research, such as, but not limited to: 1) ethical considerations, 2) the unreliable self-reporting by takers, 3) variables that are difficult to control in human studies that include nutrition and genetics. 4) Other drugs that might be used during the study and that may interfere with the results of the study. 5) Insufficient samples. Therefore, the choice of an animal model that would effectively address these questions is necessary. With animal models the conditions can be easily controlled. However there is no single species that would be exactly the same as humans. Differences between any potential animal model and humans need to be taken into consideration in order to design experiments to effectively investigate developmental toxicities. Although rats and mice are the most widely used animal models for this type of research, the use of fish models, particularly zebrafish and
Japanese medaka, is currently emerging as an alternative to mammalian models because of their low maintenance cost, short life cycle, and easy accessibility to the study of gene function (Rico et al., 2007). Non-mammalian animal models, especially fish models, have the additional advantages of having simple nervous systems and shorter generation intervals. Fertilization and embryonic development in most fish is external (in the outside environment), which eliminates placental-fetal influences. Moreover, fish embryos are covered by a transparent chorion which allows direct observation of the embryos inside without interfering with embryogenesis. Transgenic fish have proven to be very good tool for screening genes and active molecules that have regulatory roles in the biological system. One of the goals of this investigation is to induce some of the neurological deficits exhibited in FASD individuals in medaka fish as an animal model. This would allow for further investigation and understanding of the disease and provide means for possible drug development.

1.4 Japanese medaka (*Oryzias latipes*)

The Japanese medaka (*Oryzias latipes*) is classified into the family Adrianichthyidae and genus *Oryzias* they are also known as Japanese ricefish. It is a small fish (2-4 cm long) native to Southeast Asia. It moves between salt and freshwater at some point in its life. It is found in both ocean and river habitats. The female carries her eggs attached between her anal fins. Japanese medaka lives in a peacefully mixed aquarium. The generation time is 2 to 3 months, which is comparable to that of zebrafish and mice. They have a genome size, of about 800Mb, which is about one-half of the zebrafish genome. This makes it easier for gene sequencing (Heaton et al., 2003).
1.4.1 Experimental features of Japanese medaka

Medaka fish present experimental features that are unique among vertebrates. Japanese medaka is the only known non-mammalian vertebrate that has two sex determining genes, Sry and Dmy. Fish bones share many cellular and morphological aspects with mammals (Renn et al., 2006, and Willems et al., 2012). Moreover teleosts, such as medaka and zebrafish are particularly suitable for developmental studies of the brain. The structural anatomy of the head skeleton of these two fish species is a little different. The trabecular cartilages (TC) in the neurocranium of zebrafish hatchlings are fused caudally with the polar cartilages (PC), however, in medaka the caudal ends of the TC remain separated from the PC (Langille and Hall, 1987). This feature in medaka allows for the study of each bone individually. Furthermore during neurogenesis the overall pattern of the longitudinal fiber systems in medaka brain is more like that of mouse, than that of zebrafish (Ishikawa and Yuji, 1997, Ishikawa, 2004). Matsui et al. 2012 used medaka as an animal model for Parkinson's disease. They generated their medaka models using genetic manipulation or toxin exposure. They concluded that they have been able to develop for the first time a PD fish model that works both by toxin exposure and by induction of mutation. The cardiovascular morphology of the zebrafish embryo differs from mammals during development, but medaka embryos follow the common vertebrate embryonic circulatory patterns (Fujita et al., 2006). Matsumoto et al. 2009 conducted a simple screening of nonsynonymous SNPs for 11 genes that have apparent orthologous between human and medaka. They found SNPs in medaka at the same sites as in humans. They conclude that medaka is a promising model system for comparative genomics and that it could also be used for testing
variation in drug response in humans. Based on these findings, medaka embryos can be used as an experimental system that closely mimics the human system for investigating the mechanisms of alcohol teratogenesis during development and to test for preventive agents as well.

1.4.2 Using medaka as animal model for FASD

Using different dosing regimens and exposure times during embryonic development, we should be able to replicate some of the FAS related features in animal models (Becker et al., 1996). The pharmacologically relevant BACs should lie between 20 -170 mM, to approximate to different rates of drinkers (Pantazis et al., 1992). Previously in our laboratory it was demonstrated that medaka embryos, when exposed to 300 mM alcohol for 48 hour 0-2 and 1-3 day post fertilization, produced craniofacial, skeletal, and cardiovascular abnormalities, which are analogous to the FAS phenotypic features observed in human (Hu et al., 2009). In this study we have tested the possibility of inducing FASD like neurologically relevant deformities in medaka embryos as well as testing for curative agents from a natural product “Asian ginseng”.
CHAPTER 2

CENTRAL HYPOTHESIS OF THE STUDY AND SPECIFIC AIMS

Part 1: Embryo Exposure

Hypothesis

When Japanese medaka embryos are exposed during development to alcohol the alcohol is metabolized by the alcohol metabolizing enzymes generating oxidative stress. Oxidative stress leads to the formation of reactive oxygen species (ROS) superoxide anion radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), which are the precursors of the highly reactive hydroxyl radical (OH). Once formed, hydroxyl radicals interact with carbohydrates, proteins, lipids and nucleic acids to form peroxy radicals (ROO$^-$) as intermediates that can propagate damage to biomolecules to produce cell damage and/or death. Asian ginseng (PG), is one of the best-selling botanicals in the USA for many years, has been shown in a number of studies to have anti-alcoholic activities in mice and rats by augmenting their antioxidant activities. Moreover, consuming Asian ginseng during pregnancy has not been reported to cause any adverse effect to the fetus. In this study we used (PG) methanolic root extract also known as black ginseng to investigate its ability as a preventive agent of fetal alcohol spectrum disorder using Japanese medaka as an animal model. We hypothesize that alcohol is causing FASD-like effects in the
embryos by generating oxidative stress and that Asian ginseng will prevent that effect by augmenting antioxidant activities of the embryos.

Figure 1: Proposed protective effect of ginseng against alcohol toxicity
Specific Aims

**Aim 1: Determine if Asian ginseng (PG) is able to attenuate alcohol-induced, molecular and morphological, changes in Japanese medaka embryo**

**Rationale:** We investigated the possibility of developing a drug from a natural product (PG) to protect against fetal alcohol toxicity using Japanese medaka as an animal model. Most anti-alcoholic drugs are pregnancy category C (adverse effects on the fetus in animal studies but no human trials) and therefore cannot be used during pregnancy and cannot be tested for FASD.

1.1 Determine the lethality of PG root extract on Japanese medaka embryogenesis.

1.2 Determine the effects of PG as a supplement to alcohol treatment in the prevention of alcohol teratogenic effect on circulation, hatching efficiency, and neurocranium cartilage development in Japanese medaka embryos.

1.3 Determine the effect of PG on embryonic alcohol concentration in Japanese medaka embryos.

1.4 Determine the effects of PG on alcohol-induced alteration of alcohol metabolizing enzymes (adh5, adh8, aldh2, and aldh9a) and oxidative stress enzymes (*catalase*, *glutathione reductase (GR)*, *and glutathione-S-transferase,(GST)*) mRNA content in Japanese medaka embryos.

**Aim 2: Measure the concentration of the ginsenosides in the different experimental media we used in this study**

The PG stock solution alone and with the addition of alcohol to the medium was analyzed in concentrations corresponding to those used in the actual exposures. This helped to further understand the mechanism of action by which PG is producing the observed effects.
Rationale: Asian ginseng crude extract contains a number of compounds called ginsenosides, which are known to give ginseng its characteristic activity. In this study we have found a biphasic dose dependent effect of ginseng when added as a supplement to alcohol 300mM exposure in early embryonic development. Therefore we analyzed the PG stock solution alone and with the addition of alcohol to the medium in concentrations corresponding to those used in the actual exposures. We aimed at testing for any change in the concentration of ginsenosides, and increase or decrease in the availability of ginsenosides in solution, due to addition of alcohol to the media, which may help to explain the biphasic nature of activity that we are getting.

Aim 3: Compare the effects of antioxidants e.g., N-acetyl-cysteine and ascorbic acid with that of PG in the protection of medaka embryos from alcohol induced morphological changes.

Rationale: We are using these compounds as positive controls in order to verify that the protective effects that we are getting with PG could be through an antioxidant mechanism.

Aim 4: Investigate the possibility of inducing neural cell changes characteristic of FASD in Japanese medaka embryos and the effect of PG on these changes.

1.1. Determine Purkinje cell number and size as being the most susceptible neural cell for alcohol toxicity, in control, alcohol alone, PG alone, and alcohol + PG treated embryos.

Rationale: in previous work, our lab has induced some cardiovascular and craniofacial cartilage deformities related to FASD in medaka as an animal model. Brain damage, which represents a very important part of FASD, was not characterized. Alcohol induced brain damage can occur
with or without the facial and cardiovascular features. The cerebellum is vulnerable to growth restriction and neuronal depletion induced by alcohol exposure during the brain growth spurt (Heaton et al., 2006, and Goodlett et al., 1991). In this study we will try to induce neural cell changes characteristic of FASD in medaka, and investigate whether or not PG will be protective to the embryo against these changes.

1.2. Measure neuroligin (nlgn) gene mRNA expression pattern in control, alcohol alone, PG alone and alcohol + PG treated embryos.

Rationale: To investigate the possible alterations in the nlgn gene expression by direct embryonic alcohol exposure. The nlgn gene has been implicated in autism and other cognitive disorders that are also found in FASD. It is responsible for induction of contact-mediated presynaptic differentiation in contacting neurons and it has been found to play a role in angiogenesis. We will also investigate whether or not PG will be protective to the embryo against these alterations.
Part II: Adult Fish

Hypothesis

Trans-generational epigenetic inheritance is a well-documented phenomenon in plants and fungi. It was not convincingly demonstrated in mammals until recently (Cavalli et al., 1998, V. Rakyan, and E. Whitelaw 2003, Marie-Theres Hauser et al., 2011). Epigenetic regulation of the agouti viable allele (Avy) in isogenic mice caused alteration in coat color and obesity/diabetes like phenotype. This aberrant phenotype was transmitted to the next generation via an epigenetic modification in the female germline (Wolff et al., 1998). In the last few years a link has emerged between alcohol-induced epigenetic modifications and behavioral and phenotypic changes in humans. However, few studies have addressed whether or not these epigenetic changes can be transmitted to the offspring, although offspring of alcoholics showed cognitive, physiological and psychological problems with increased risk for developing alcoholism. Also there is a probable link between alcohol consumption by men with increased demethylation of normally hypermethylated-imprinted regions in the sperm DNA, and the development of FASD is being investigated (Ouko et al., 2009). In this study, we are chronically exposing the adult Japanese medaka, both male and female, to alcohol and looking for epigenetic changes that could be transmitted to the offspring. We hypothesize that some epigenetic changes can occur due to chronic alcohol exposure, and that these changes are transmitted to the offspring leading to some of the neurobehavioral and morphological deformities shown in FASD.
Specific Aims

Part II

In this part of the study we investigated embryo susceptibility toward developing FASD features after chronic alcohol exposure of the parent fish, female and male.

Aim 1: Parent:

1.1 Determine the effect of chronic alcohol exposure on egg production and fertilization of reproductively active female fish chronically exposed to alcohol.

1.2 Determine the effect of chronic alcohol exposure on alcohol metabolizing and oxidative stress-related enzymes in ovary and liver of reproductively active female fish.

Aim 2: Embryo

2.1 Determine the effect of parental alcohol exposure on vessel circulation status of medaka embryogenesis.

2.2 Determine the effect of parental alcohol exposure on neurocranial cartilage development in medaka embryogenesis.

Rationale: To investigate the possibility of inheritance of epigenetic changes from the alcohol exposed parents to their offspring and the development of FASD like morphological deformities.
CHAPTER 3

MATERIALS AND METHODS

3.1 Embryo culture

IACUC approved methods of animal maintenance; egg collection and embryo culture conditions were previously described (Hu et al., 2008, 2009). In brief, fertilized eggs after collection were maintained in hatching solution (17 mM NaCl, 0.4 mM KCl, 0.3 mM MgSO₄, 0.3 mM CaCl₂, with the required amount of NaHCO₃ to maintain the pH at 7.4 and 0.0002% methylene blue to reduce fungal infection) in a precision high performance incubator (Thermo Fisher Scientific, Waltham, MA) at 26±1 °C with 16L: 8D light cycle. PG was initially dissolved in DMSO and diluted to desired concentration with hatching solution keeping the final DMSO concentration at 1μl/ml (0.1%). PG (50-200 μg/ml) and alcohol (300 mM) was added to the medium after transferring the viable embryos at respective developmental stages to 2 ml tubes (1 egg/ tube in 1 ml medium). To stop alcohol loss by evaporation the tubes were tightly capped. The DMSO concentration in both control and alcohol treated embryos was adjusted to 0.1%. Embryos were exposed to different treatment conditions at two different time points of development (A group: 0 dpf, blastula, Iwamatsu stages ~ 9-10, and B group: 1 dpf, neurula, Iwamatsu stages ~17-20) and discontinued at 48 h, following a onetime renewal of media after
24 h. Embryos, before sacrifice, were examined for routine developmental changes (blood clots, and vessel circulation) under a phase contrast microscope (AO Scientific Instruments) and were classified after Iwamatsu (2004).

3.2 Preparation of the ginseng

Asian ginseng (*Panax ginseng*) roots used in these experiments were collected from China and after identification; vouchers (voucher number 66) were stored in the National Center for Natural Products Research (NCNPR) repository for reference. The collected materials, which were free from insects, diseases, and bryophytes, were washed several times with distilled water, cut into manageable pieces, and kept warm (below 40°C) until thoroughly dry. The dried material (one mm mesh size) was weighed and packaged in 950 ml opaque amber high-density polyethylene (HDPE) wide-mouth jars with an identification label. The jarred samples were deposited into the NCNPR plant material storage facility until extraction. For extraction, 7.5 g of powdered plant material was used in an automated process utilizing programmed Dionex Accelerated Solvent Extraction (ASE200/300) systems. The extraction was made using 95% methanol at 40°C and 1,500 PSI for 10 min. This process was repeated twice more to afford the final pooled extract. This extract was concentrated using a combination of rotary evaporation and vacuum centrifugation. All pertinent information regarding the extraction and the representative plant material was placed on the vial containing the dried extract, and the samples were maintained at -80°C. The concentrated extract was used for TLC, LC/MS, and HPLC analysis for chemical composition.
3.3 Embryo exposure

Fertilized eggs were incubated in hatching solution in tightly caped eppendorff tubes (1 egg/tube in 1 ml hatching solution) with or without (300 mM) of alcohol or ginseng (50-200 µg/ml) or both for 48 h (0-2 or 1-3 dpf). After incubation embryos were transferred to 48 well plates (1 egg/well/1 ml hatching solution) in clean hatching solution. Embryo mortality and deformity was noted daily and the dead embryos were removed from the experiment. Each experimental group consisted of 8-10 embryos and each experiment was repeated 3-4 times.

3.4 Cartilage staining

Embryos were stained in 1% Alcian blue (Sigma-Aldrich). Embryos 6-7 dpf were anesthetized in MS 222 (Sigma-Aldrich) (0.1 % in hatching solution) then fixed in 4% paraformaldehyde (Sigma-Aldrich) in phosphate buffer saline (PBS) + 0.1% Tween 20 (PBT) for 4-6 h. Embryos were decorionated and refixed in 4% paraformaldehyde + 0.1% Tween 20 for 24h at 4°C. Fixed samples were washed twice in nano pure water then treated with 5% TCA (Sigma-Aldrich) for 10 minutes. Samples were then washed once with acid ethanol solution (0.37% HCl, 70% alcohol) before being stained with 1% Alcian blue for 4 hours. Excess stain was removed by 2-3 washings with acid ethanol and samples were transferred to hydrogen peroxide (1%) for clearing overnight. Trypsin (Sigma-Aldrich) was added to the samples for muscle digestion after removal of hydrogen peroxide for 4-6 h. Digital images of Head skeleton from various groups were captured using Olympus B-max 40 microscope.
3.5 Determination of embryonic alcohol concentrations

Alcohol concentrations in the embryos exposed either to alcohol or alcohol and PG were determined after Reimers et al (2004) with some modifications (Wang et al., 2006). In brief, group A embryos (Iwamatsu Stages 9-10) in 2 ml tubes were exposed to one ml of 300 mM alcohol with or without 100 μg/ml PG for 48 h. The media were changed once at 24 h. Parallel control embryos were also maintained in clean hatching solution (no alcohol or PG) or containing only PG (100 μg/ml). After removal from culture, the embryos were transferred to 1.5 ml centrifuge tubes on ice and washed with 1 ml cold 3.5% perchloric acid (PCA) twice to remove residual alcohol from outside of the chorion. Four embryos from each group were pooled together and homogenized in 100 μl 3.5% PCA. The homogenate was centrifuged at 12000Xg for 15 min at 4°C. The supernatants were saved and stored at 4°C in sealed tubes. The alcohol concentration of the supernatants was determined in a 96-well plate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA, USA) at 340 nm by measuring NADH production at 37°C as follow. The reaction mixture in 200 μl final volume contained 175 μl NAD in 0.5M Tris pH 8.8, 15 μl yeast ADH (0.75 mg/ml) and 10 μl of either sample or standard alcohol (44-221 μg) solution. After 2 min pre-incubation at 37°C, production of NADH was measured. A blank reaction without alcohol (10 μl 3.5% PCA) was run simultaneously to correct for any substrate-independent generation of NADH. In this condition, the linearity of the reaction was maintained for ~15 min. and embryonic alcohol concentrations were determined by comparison with standard curves. The results were expressed as mg alcohol/egg or calculated to mM alcohol considering the average diameter of the egg to be 1200 μm.
3.6 Cloning

E. coli (JM109) cells were infected with pGEM T easy vector and ligated with the target DNA fragment. They were chilled on ice for 30 min then heat shocked at 37°C for 30 seconds and put back on ice for 10 minutes. LB medium was added (up to 1ml) the cells were then incubated in a shaker incubator at 37°C, 200 rpm for 6 hours. X-gal and IPTG were spread on LB agar plates with ampicillin for at least 30 minutes at room temperature. Cells were centrifuged and most of the supernatant was discarded leaving around 100 μl of the medium to be used for resuspension and then spreading on the plates. Plates were incubated upside down at 37°C overnight (12-16 hrs). Plates were stored at 4°C until used.

3.7 Quantitative real-time RE-PCR (qPCR)

The mRNA content of alcohol metabolizing enzymes (adh5, adh8, aldh1A2, aldh 2, and aldh9a) and the oxidative stress related enzymes (catalase, GST, and GR) were determined by qPCR (Hu et al., 2008; Wu et al., 2008). In brief, total RNA of group A embryos were extracted on 6 dpf by Trizol reagent following the manufacturer’s instructions and the genomic DNA was removed from RNA by treating with DNase I (Dasmahapatra et al., 2000). RNA was reverse transcribed to cDNA (Biorad) and 1 μl of the cDNA was used for qPCR analysis. Gene-specific primers (Table 1) used for target gene amplification were verified and the relative quantity was determined considering Elongation Factor α 1(EFα1) as internal control.
3.8 Paraffin embedding, sectioning and staining

Samples (brain, liver and ovary) were fixed in 4% PFA in PBS (PH 7.4) for 2 days with one change at 24 h. This was followed by dehydration in increasing gradients of alcohol (70-100%). After clearing in Clearify™ (American Master Tech Scientific, INc., Lodi, CA), they were embedded in melted Paraffin (Parablast, Fisherbrand™) at 60°C. Then they were sectioned at 7 µM thickness using a microtome. Sections were cleared using xylene and rehydrated in alcohol series of (30-100%) followed by staining either with Hematoxylin (liver and ovaries) or crystal violet (brain) and counter stained with eosin. Slides then were mounted and covered with cover slips for investigation under light microscopy. Pictures were taken using a digital camera with element software.

3.9 Adult exposure

Adult medaka fish either female only or both male and female were exposed in a 1 L bottle (1 L Filter system bottle) to 300 mM alcohol each day in the afternoon from 2:00 pm until they start to lay on one side or flip over or up to 2 hours, whichever happened first. Bottles were filled with 400 ml with the exposure media (hatchling solution for control or alcohol solution for treatment). Each bottle contained 1 female for the female only treated group and 1 female and 1 male fish for the both treated group. One week before the start of the actual exposure fish were acclimated for the bottle treatment by putting them in the bottles with only hatching solution for all groups each day for 2 hours at the same time as exposure time.
3.10 Egg collection and examination

Eggs were collected from each female in each group separately and examined under light microscope for the number of fertilized eggs, and unfertilized eggs. The eggs then were maintained in normal conditions until hatching. Circulation, hatching efficiency and TC and NC cartilage were examined. Comparison of the overall production difference was assessed between the treatment group and the control group as eggs /d/g body weight.

3.11 Statistical analysis and data interpretation

Results were analyzed using GraphPad Prism 5.0 software. Substance-dependent (alcohol, ginseng), and (morphological deformities, mRNA copy) data was presented as mean ±S.E. Between groups statistical differences were determined using one way ANOVA followed by post-hoc Tukey’s multiple comparison test (p < 0.05) or student t test (p < 0.05).
CHAPTER 4

RESULTS

Part 1: Medaka embryo

Aim1: Determine if Asian ginseng (PG) is able to attenuate alcohol induced morphological and biochemical features in Japanese medaka embryogenesis.

Rationale: The use of PG as a preventive agent of FASD was mainly due to the fact that the use of PG protected against alcohol teratogenesis in mouse embryo by augmenting its antioxidant defense (Lee et al., 2009). Furthermore PG was able to prevent alcohol-induced reduction of neonatal brain weight in rats (Okamura et al., 1994). We hypothesize that ginseng would be able to protect medaka embryos against alcohol-induced morphological features during Japanese medaka embryogenesis.

1.1. Determine the lethality of ginseng root extract on medaka embryogenesis:

To determine the lethal concentration of PG on medaka embryogenesis, fertilized medaka eggs A group (0-2dpf) or B group (1-3dpf) were exposed to different concentrations of PG (0-2000 μg/ml) for 48 h and then transferred to clean hatching solution. Mortality was evaluated on 10 dpf. The calculated LC$_{50}$ values for PG to cause 50% mortality in embryos at 10 dpf as determined from three independent experiments was 355.5 ±1.12 μg/ml in A group and 679.6 ±
1.6 μg/ml in B group with 0-48 hpf constant exposure followed by clean hatching solution. Embryo survivability and hatching was severely affected at concentrations > 500 μg/ml with 100% mortality for group A and >750 μg/ml for group B (Figure 2). Removal of PG from the medium allowed the surviving embryos to initiate vessel circulation that was found to be dose-dependent on the PG concentration. The majority of the embryos exposed to 250 μg/ml PG were late in the initiation of vessel circulation (~3 dpf) compared to the controls and 125 μg/ml (~2 dpf) groups. However, all the surviving embryos (≤ 250 μg/ml) were able to start vessel circulation by 6 dpf. Hatching of the surviving embryos was also affected by PG in a dose-dependent manner (Figure 3) and related to the vessel circulation status of the embryos. In our laboratory conditions the embryos generally started to hatch on 7+ dpf (~175 hpf) and if the embryos were unable to hatch by 14 dpf, we considered them as unhatched. The hatching efficiency of the embryos as determined on 10 dpf is dependent upon the concentration of PG in the medium, especially in B group (Figure 3B). Of the embryos that were able to initiate vessel circulation by 6 dpf majority of them in both A and B groups had hatched by 10 dpf (the concentration of PG ≤250 μg/ml). Only 5-20 % of the surviving embryos in B groups treated with 500-750 μg/ml PG were able to hatch if they had vessel circulation while the ones that did not initiate vessel circulation by 6dpf were not able to hatch by 14dpf and were considered unhatched (Figure 3B). From these initial experimental data we have set up the maximum limit of PG concentration to be 200 μg/ml in all other experiments.
Figure 2: *Panax* ginseng mediated mortality in medaka embryos. The LC$_{50}$ of ginseng in medaka embryos 0-2 dpf A group (A) and 1-3 dpf B group (B) as determined from five independent experiments is 355.5 ± 7.03 μg/ml and 679.6 ± 1.6 μg/ml respectively.
Figure 3: Hatching of the surviving embryos was affected by PG in a dose-dependent manner and related to the vessel circulation status of the embryos; n=8, one-way ANOVA followed by Tukey’s multiple comparison tests (*) = p < 0.05.
1.2. Effects of PG as a supplement to alcohol teratogenesis.

Medaka embryos at two stages of development (group A: Iwamatsu stages 9-10, and group B: Iwamatsu stages 17-18) were exposed to alcohol (A300 mM) with or without sub-lethal concentrations of PG (50-200 μg/ml), parallel controls were maintained in clean hatching solutions with or without PG for 48 h. Embryos were then transferred to clean hatching solution with no alcohol or PG. All of A group embryos died by 2 dpf when exposed to alcohol (A300 mM) simultaneously with 200 μg/ml PG (Figure 4). When PG concentration was reduced to 50-100 μg/ml with 300 mM alcohol embryo survivability enhanced and more than 50% of these embryos (A group) were viable even though the viability was significantly less than the corresponding control and PG (100 μg/ml)-treated groups. In B group, the embryo survivability in these conditions (A300 mM + 50-100 μg/ml PG) was equal to the embryos maintained in a clean (controls) hatching solution (Figure 4). The vessel circulations of the surviving embryos were examined on 6 dpf. It was observed that A group embryos when exposed to alcohol (A300 mM) alone or in combination of PG (50 and 100 μg/ml), the vessel circulation was found to reduce significantly in alcohol 300 mM only and alcohol 300 mM + PG (50 - 100 μg/ml) in comparison to controls or to the embryos maintained only in PG (100 μg/ml) (Figure 5A). However, in B group embryos the combination of alcohol (300 mM) and PG (50-200 μg/ml) showed dose-dependent improvement in the embryo’s circulation compared to controls and embryos exposed to only PG (200 μg/ml). Reduced vessel circulation was only seen in those embryos exposed to only alcohol (300 mM) or alcohol (300 mM) + PG (200 μg/ml). Alcohol (300 mM) in combination with PG concentration of (50 - 100 μg/ml) resulted in vessel circulations of the embryos that were at the same level as in controls or in the embryos exposed
to only PG (200 μg/ml) (Figure 5B). The HE of the embryos was also examined at 10 dpf in these culture conditions. It was observed that like vessel circulation, A group embryos when exposed to only alcohol (300 mM) or alcohol 300 mM in combination with PG 50-100 μg/ml HE was reduced significantly when compared with the controls and the embryos exposed to only PG (100 μg/ml) (Figure 5A). For group B embryos, there was no significant difference in HE between any of the groups (Figure 5B).

Figure 4: Effect of simultaneous exposure of PG and alcohol on mortality of medaka embryos during development. Group A embryos were treated 0–2 dpf and group B embryos were treated 1–3 dpf. Mortality was examined on 10 dpf. n = 5–10, one way ANOVA followed by Tukey’s multiple comparison test (*) or (#) p < 0.05.
Figure 5: Effects of alcohol and PG on the onset of circulation in medaka embryogenesis exposed 0-2 dpf (A group) and 1-3 dpf (B group). It was observed that A group embryos when exposed to alcohol (300 mM) alone or in combination with PG (50 and 100 μg/ml), the vessel circulation was found to be reduced significantly in comparison to controls or with the embryos maintained only in PG (100 μg/ml). Alcohol (A300 mM) only or in combination with PG (50 - 100 μg/ml) did not cause any significant difference in this group. However, in B group combinations of alcohol (A300 mM) and PG (50-200 μg/ml) showed dose-dependent improvement in embryo’s circulation compared to controls and embryos exposed to only PG (200 μg/ml). Reduced vessel circulation was only seen in those embryos exposed to only alcohol (A300 mM) or alcohol (A300 mM) + PG (200 μg/ml); n=8, one way ANOVA followed by Tukey’s multiple comparison test, p < 0.05.
Figure 6: Effects of alcohol and PG on hatching efficiency in medaka embryogenesis exposed at 0-2 dpf and 1-3 dpf. The hatching efficiency of the embryos was also examined at 10 dpf in these culture conditions. It was observed that like vessel circulation, group A embryos when exposed to only alcohol (300 mM) or in combination of alcohol and PG (50-100 μg/ml) HE was reduced significantly when compared with the controls and the embryos exposed to only PG (100 μg/ml)(A). For group B embryos, there was no significant difference in HE between any of the groups (B); n=8, one way ANOVA followed by Tukey’s multiple comparison test (*) = p <0.001.
Embryos belonging to group B on 6-7 dpf were further examined for TC development after staining with Alcian blue (Figure 7). Control embryos have paired TC located between the anterior end of the hyosymphetic and the rostral end of the parasphenoid bone (nonspecifically stained in Alcian Blue) with extended distal ends lying just above the proximal end of the polar cartilages. The embryos exposed to only PG (200 μg/ml) have well developed TC. The embryos exposed to only alcohol (A 300 mM) have developed tiny TC structures on 7 dpf that remained adjacent to the ethmoid plate. Further examination indicates that the linear length of TC of alcohol + PG group embryos was comparatively longer than those exposed to alcohol alone (Figure 8A). The linear length of the neurocranium and TC of group B embryos on 7 dpf exposed to alcohol (A 300 mM) only, alcohol (A 300 mM) with various combination of PG (50-200 μg/ml) or PG only were measured and compared with control embryos on 7 dpf. It was observed that the linear length of the neurocranium was significantly reduced in all treatment groups other than only PG (200 μg/ml) when compared with control embryos (Figure 8B).
Figure 7: Representative photomicrographs of Alcian blue stained group B medaka embryos showing trabecular cartilage development in the neurocranium on 7 dpf. The photomicrograms were taken in an Olympus B-max 40 microscope at constant magnification. Both control, PG, and alcohol 300 mM and PG 50-200 μg/mL treated embryos showed well-developed trabecular cartilages (TC) and polar cartilages in the neurocranium, whereas alcohol 300 mM treated embryos had tiny TC.
Figure 8: The trabecular (TC) (A) and neurocranial (NC) (B) cartilage lengths in 7 day embryos treated for 48 h 1-3 dpf. TC is significantly decreased in size in alcohol 300 mM and alcohol 300 + PG 200 µg/ml groups but not in A300+P50 µg/ml. These data show that PG 50 µg/ml is able to attenuate alcohol toxicity as manifested by decreased length of the trabecular cartilage. Regarding the whole neurocranium PG was not able to restore the neurocranium cartilage length back to control size; n = 6-8, one-way ANOVA followed by Tukey’s multiple comparison test $p < 0.05$. 
1.3 Determine PG effect on embryonic alcohol concentration in medaka embryos.

To determine whether PG could change the alcohol permeability of the chorion in Japanese medaka embryogenesis, we measured the embryonic alcohol concentration of the embryos after exposing them to alcohol (300 mM) with or without PG (100 μg/ml) for 48 hpf.

Parallel embryos were run without alcohol (control) or with PG (100 μg/ml) and used as negative controls. It was observed that the embryos absorbed a substantial amount of alcohol if it was added to the medium; however, negative data (A\text{340}) were obtained in the absence of alcohol (Figure 9), which might be a result of post enzyme-substrate reactions. The average calculated alcohol concentration in embryos exposed to only alcohol is 95 mM, which is approximately 32% of the 300mM alcohol concentration added to the medium. Addition of 100 μg/ml PG to the medium did not significantly change the embryonic alcohol concentration (average alcohol concentration is 84 mM which is almost 28% of the 300mM alcohol added to the medium) of the embryos when compared with the embryos exposed to only alcohol.
Figure 9: Determination of embryonic alcohol concentration of medaka embryo. The embryonic alcohol concentration after A 300 mM or A 300 mM + PG 100 μg/mL waterborne exposure for 48 h was estimated and compared to controls and PG only groups using an ADH-dependent kinetic assay following Reimers et al. (2004) (A). The volume of the embryos was calculated on the basis of an average embryonic diameter 1200 μm, no significant difference in the embryonic alcohol concentration was detected between alcohol only group and alcohol + PG group(B); n = 4 pools, pool = 4 embryos, student t test p < 0.05.
1.4 Determine PG effect on alcohol metabolizing enzymes (*adh5, adh8, aldh2, and aldh9a*) and oxidative stress enzymes (*catalase, glutathione reductase (GR), and glutathione S-transferase (GST)*) mRNA contents in medaka embryos.

We have measured the effects of alcohol and PG combined embryonic exposure at 0-2 dpf on the expression patterns of alcohol metabolizing enzyme (*adh5, adh8, aldh2 and aldh9*), and oxidative stress enzymes (*catalase, GR, GST*) mRNAs.

One of the potential mechanisms of alcohol toxicity is the induction of oxidative stress due to alcohol metabolism. According to Lee et al., (2009) ginseng was able to protect mouse embryos against alcohol toxicity through augmentation of antioxidative mechanisms. We therefore focused on mRNAs of alcohol metabolizing enzymes (*adh5, adh8, aldh2, aldh9*) (Figures 10 and 11) and enzymes related to oxidative stress (*catalase, GR, GST*) (Figure 12). Among these enzymes *adh8, aldh9a, and GST* are developmentally regulated. Our results showed that alcohol alone was able to reduce significantly the mRNA content of *adh8 and GST* in comparison with controls. However, other mRNAs remained unaltered. Adding PG simultaneously with alcohol during early embryonic exposure did not protect the embryos against the mRNA changes in alcohol metabolizing enzymes nor oxidative stress enzymes induced by alcohol exposure alone.
Figure 10: Effect of PG (100 μg/mL), Alcohol (300 mM) and PG (50–100 μg/mL) + alcohol (300 mM) on adh5 (A) and adh8 (B) mRNA content of medaka embryos (group A) 6 dpf. Total RNA was prepared from 6 to 8 pooled embryos and reverse transcribed and analyzed by qPCR. For each sample, the threshold cycle for internal standard (EFα1) amplification (Ct, EFα1) was subtracted from the threshold cycle of the corresponding enzyme mRNA amplification (Ct, enzyme) to yield ΔCt. For each treatment group, the data are the mean of ΔCt of control samples, which was subtracted from each individual sample to yield individual ΔΔCt. Fold induction relative to control samples was calculated as $2^{-\Delta\Delta Ct}$. n = 3–4, one way ANOVA followed by Tukey’s multiple comparison test, p < 0.05.
Figure 11: Effect of PG (100 μg/mL), Alcohol (300 mM) and PG (50–100 μg/mL) + alcohol (300 mM) on aldh2 and adh9 mRNA content of medaka embryos (A group) 6 dpf. Total RNA was prepared from 6 to 8 pooled embryos and reverse transcribed and analyzed by qPCR. For each sample, the threshold cycle for internal standard (EFα1) amplification (Ct, EFα1) was subtracted from the threshold cycle of the corresponding enzyme mRNA amplification (Ct, enzyme) to yield ΔCt. For each treatment group, the data are the mean of ΔCt of control samples, which was subtracted from each individual sample to yield individual ΔΔCt. Fold induction relative to control samples was calculated as $2^{-ΔΔCt}$. n = 3–4, one way ANOVA followed by Tukey’s multiple comparison test, p < 0.05.
Figure 12: A group embryos were further examined for the concentration of oxidative stress-related enzyme (catalase (A), GR (B), GST (C)) mRNA. PG (100 μg/ml) alone or in combination with alcohol (300 mM), had no effect in altering catalase, GR, and GST mRNA contents of the embryos in all alcohol treatment groups when compared with controls; n = 3-4 pools, pool = 6-8 embryos, one way ANOVA followed by Tukey’s multiple comparison test p < 0.05.
Aim 2: Measure the concentration of the ginsenosides in the different experimental media used in this study

We have analyzed the PG stock solution alone and with the addition of alcohol to the medium in concentrations corresponding to those used in the actual exposures (Figures 13 and 14) and (Table1). We were able to further understand the mechanism of action by which PG is producing the observed effects.

Rationale: Asian ginseng root extract contains a number of compounds called ginsenosides, which are known to give ginseng its characteristic activity. In this study we have found a biphasic dose dependent effect of ginseng when added as a supplement to alcohol 300 mM exposure in early embryonic development. Therefore we analyzed the PG stock solution alone and with the addition of alcohol to the medium in concentrations corresponding to those used in the actual exposures. We aimed at testing for any change in the concentration of ginsenosides (increase or decrease the availability of ginsenosides) due to addition of alcohol to the media, which may help to explain the biphasic nature of activity that we have observed.
HPLC Chromatograms- Ginseng

HPLC-UV analysis at 205 nm [Ginsenoside Rg1 (1), Ginsenoside Re (2), Ginsenoside Rf (3), Ginsenoside Rb1(4), Ginsenoside Re (5), Ginsenoside Rb2 (6) and Ginsenoside Rd (7)]

66:10-25-2002:PAGIA Panax ginseng roots (NCNP) 502.1mg

66:10-25-2002 P. ginseng extract

Figure 13: Typical HPLC chromatograms of plant material and plant extract using UV detector at 205 nm (1) ginsenoside Rg1, (2) ginsenoside Re, (3) ginsenoside Rf, (4) ginsenoside Rb1, (5) ginsenoside Re, (6) ginsenoside Rb2, and (7) ginsenoside Rd.
Figure 14: HPLC analysis showing the separation of the different ginsenosides present in the different types of media that were used in this study in comparison to a standard ginseng root extract mix. There was almost no difference between ginsenosides concentration in the solutions with and without alcohol regarding the intensity and time of separation shown by the different peaks.
Table 1: Types of the different ginsenosides present in the different exposure media that were used in this study in comparison to a standard ginseng root extract.

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<th>Compounds</th>
<th>RT</th>
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<th>5.4</th>
<th>7.2</th>
<th>8.2</th>
<th>8.9</th>
<th>9.3</th>
<th>9.97</th>
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<tr>
<td>56 P. ginseng</td>
<td>845</td>
<td>991</td>
<td>799/845</td>
<td>1108</td>
<td>1078</td>
<td>1078/1123</td>
<td>946/991</td>
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<tr>
<td>66 P. ginseng</td>
<td>1 (P. Ginseng 200 µg/ml)</td>
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<td>2 (P. Ginseng 200 µg/ml + alcohol 300 mM)</td>
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<td>3 (P. Ginseng 50 µg/ml)</td>
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<td>4 (P. Ginseng 50 µg/ml + alcohol 300 mM)</td>
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Aim 3: Compare the effects of antioxidants e.g. N-acetyl-cysteine and ascorbic acid with that of PG in the protection of medaka embryos from alcohol induced morphological changes (Figures 15, 16 and 17).

![Graph showing vessel circulation in medaka embryos](image-url)

Figure 15: Effects of alcohol, PG and anti oxidants N-acetyl cysteine and ascorbic acid on the vessel circulation at 7 dpf in medaka embryogenesis exposed at 1-3 dpf (group B). PG 200 μg/ml+ alcohol 300 mM treatment showed significant reductions in vessel circulation in comparison to controls embryos and embryos maintained in PG (200 μg/ml) only. Alcohol 300 mM only, alcohol 300 mM + (50 - 100 μg/ml) PG and antioxidant compounds (n-acetyl cysteine and ascorbic acid) groups did not show any significant reduction in vessel circulation. n=8, one way ANOVA followed by Tukey’s multiple comparison test $p < 0.05$. 

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Figure 16: Control medaka embryo at 7 dpf (A) trabecular cartilage (cartilage stained with Alcian blue) absent in A 300 mM treatment (B) (alcohol 300 mM for 48 h, 1-3dpf), but was protected when ginseng (PG) was supplemented at 50-200 µg/ml with A 300 treatments (C). Comparable results were seen when embryonic alcohol exposure was supplemented with known antioxidant compounds e.g. N-acetyl-cysteine and ascorbic acid 10-25 mM (E & F). (D) represents N-acetyl cysteine 25 mM exposure, as a positive control TC was comparable to controls.
Figure 17: Trabecular cartilage (A) and neurocranial cartilage (B) lengths in 7 day embryos treated for 48 h 1-3dpf. TC is significantly decreased in size in A 300 mM and A 300 + PG 200 µg/ml groups but was not in A 300 + PG 50 µg/ml. Regarding the whole neurocranium ginseng was not able to restore the neurocranium cartilage length back to control sizes. Comparable results were seen when embryonic alcohol exposure was supplemented with known antioxidant compounds e.g. N-acetyl-cysteine and ascorbic acid. Both compounds were able to protect the trabecular cartilage and the neurocranial cartilage length back to control size. n=8, one-way ANOVA followed by Tukey’s multiple comparison test \( p < 0.05 \).
Aim 4: Investigate the possibility of inducing neural changes characteristic of FASD in Japanese medaka embryos.

1.1. Determine Purkinje cell number and size, as being one of the most susceptible neural sites for alcohol toxicity, in control, alcohol alone, PG alone, and alcohol + PG treated embryos.

Sections of brains of adult medaka that were treated with alcohol at embryonic stages showed underdevelopment of the Purkinje layer in the cerebellum when compared with controls (Figures 18-22). The number and size of cells in the Purkinje layer were significantly reduced in comparison to controls. The Purkinje cells are more or less oval in shape so we measured the longest diameter of the cell as a marker for the size of the cells (Figures 22 and 23).
Figure 18: Control medaka cerebellum stained with crystal violet showing all 3-cell layers with the Purkinje cell layer in between the molecular and granular layers (A). (B) Higher magnification showing details.
Figure 19: Adult medaka fish cerebellum embryonically treated with 300 mM alcohol 1-3 dpf showing only 2 cell layers with the Purkinje cell layer almost completely undeveloped (A). (B) Higher magnification of Purkinje cell layer.
Figure 20: Adult medaka fish cerebellum embryonically treated with PG 50 μg/ml at 1-3 dpf showing all 3 cell layers with the Purkinje cell layer being present (A). (B) Higher magnification showing details.
Figure 21: Adult medaka fish cerebellum embryonically treated with 300 mM alcohol simultaneously with PG 50 μg/ml at 1-3 dpf showing all 3 cell layers with the Purkinje cell layer being present (A). (B) Higher magnification showing details.
Figure 22: This graph demonstrates the average number of Purkinje cells in 4 sections of each sample. The data shows that the number of cells is significantly lower in the alcohol treated group (A 300) compared with controls, PG 50, and PG 50+ A 300 groups. These results show that adding PG 50 simultaneously with alcohol exposure is able to prevent alcohol toxicity on the Purkinje cell layer; n = 4 sections, one-way ANOVA followed by Tukey’s multiple comparison test $p < 0.05$.

Figure 23: This graph demonstrates the average diameter of Purkinje cells in (10 cells/section) of each treatment group. The data shows that the diameter of cells is significantly lower in alcohol treated group (A 300) in comparison with controls, PG 50, and PG 50+ A 300 groups. These results show that adding PG 50 simultaneously with alcohol exposure is able to prevent alcohol toxicity on the Purkinje cell layer; n = 4 sections, section = 10 cells one-way ANOVA followed by Tukey’s multiple comparison test $p < 0.05$. 
1.2. Measure neuroligin ($nlgn$) mRNA expression pattern in control, alcohol alone, PG alone and alcohol + PG treated embryos.

We investigated first the normal expression pattern of different $nlgn$ (1, 2a, 2b, 3a and 3b) genes during normal embryonic development of medaka embryos. It was found that they have different patterns of expression during embryogenesis. Then we investigated the expression patterns of $nlgn$ (1, 2a, 2b, 3a and 3b) genes at 6 dpf after exposing the medaka embryos to alcohol alone and alcohol and PG at 1-3 dpf with a parallel control group with no treatment. There was no significant alteration detected in the $nlgn$ (1, 2a, 2b, 3a and 3b) genes expression pattern between the alcohol only and alcohol + PG exposed groups and the control group (Figures 24-33).
Figure 24: This graph demonstrates the mRNA expression of *nlgn 1* during normal embryonic development. It shows that highest expression is at 0 dpf. \( n = 4 \) pools, pool = 4-6 embryos, one way ANOVA followed by Tukey’s multiple comparison test \( p < 0.05 \).

Figure 25: This graph demonstrates the effects of adding alcohol alone, PG alone, and alcohol and PG together on the *nlgn 1* mRNA expression during embryonic development on 6 dpf. Even though there is a tendency for decreased mRNA expression in alcohol treated and alcohol and PG treated groups on 6 dpf, the decrease is not significantly different from controls. \( n = 4 \) pools, pool = 4-6 embryos, one way ANOVA followed by Tukey’s multiple comparison test \( p < 0.05 \).
Figure 26: This graph demonstrates the mRNA expression of *nlgn 2a* during normal embryonic development. It shows that highest expression is at 6 dpf. *n = 4* pools, pool = 4-6 embryos, one way ANOVA followed by Tukey’s multiple comparison test *p* < 0.05.

Figure 27: This graph demonstrates the effects of adding alcohol alone, PG alone, and alcohol and PG together on the *nlgn 2a* expression during embryonic development on 6 dpf. *Neuroligin 2a* mRNA expression in alcohol treated and alcohol and PG treated groups on 6 dpf, was not significantly different from controls. *n = 4* pools, pool = 4-6 embryos; one way ANOVA followed by Turkey’s multiple comparison test *p* < 0.05.
Figure 28: This graph demonstrates the mRNA expression of *nlgn 2b* during normal embryonic development. It shows that highest expression is at 0 dpf and in hatchling stage. *n* = 4 pools, pool = 4-6 embryos, one way ANOVA followed by Tukey’s multiple comparison test *p* < 0.05.

![Graph](image)

Figure 29: This graph demonstrates the effects of adding alcohol alone, PG alone, and alcohol and PG together on the *nlgn 2b* expression during embryonic development on 6 dpf. *Neuroligin 2b* mRNA expression in alcohol treated and alcohol and PG treated groups on 6 dpf, was not significantly different from controls. *n* = 4 pools, pool = 4-6 embryos; one way ANOVA followed by Turkey’s multiple comparison test *p* < 0.05.

![Graph](image)
Figure 30: This graph demonstrates the mRNA expression of \( nlgn \ 3a \) during normal embryonic development. It shows that highest expression is in hatchling stage. \( n = 4 \) pools, pool = 4-6 embryos; one way ANOVA followed by Turkey’s multiple comparison test \( p < 0.05 \).

Figure 31: This graph demonstrates the effects of adding alcohol alone, PG alone, and alcohol and PG together on the \( nlgn \ 3a \) mRNA expression during embryonic development on 6 dpf. Even though there is a tendency for decreased mRNA expression in alcohol treated and alcohol and PG treated groups on 6 dpf, the decrease is not significantly different from controls. \( n = 4 \) pools, pool = 4-6 embryos; one way ANOVA followed by Turkey’s multiple comparison test \( p < 0.05 \).
Figure 32: This graph demonstrates the mRNA expression of *nlg3b* during normal embryonic development. It shows that highest expression is in hatchling. *n* = 4 pools, pool = 4-6 embryos; one way ANOVA followed by Turkey’s multiple comparison test *p* < 0.05.

Figure 33: This graph demonstrates the effects of adding alcohol alone, PG alone, and alcohol and PG together on the *nlg3b* expression during embryonic development on 6 dpf. *Neuroligin 3b* mRNA expression in alcohol treated and alcohol and PG treated groups on 6 dpf, was not significantly different from controls. *n* = 4 pools, pool = 4-6 embryos; one way ANOVA followed by Turkey’s multiple comparison test *p* < 0.05.
Part II

Parent Exposure

In this part of our study we aimed at investigating the possibility of inheritance of epigenetic changes from the alcohol exposed parents to their offspring and the development of FASD like morphological deformities.

Medaka fish blood alcohol concentration after 300 mM alcohol exposure, was determined and our results showed that both male and female medaka achieved similar blood alcohol concentration after 2 h of alcohol exposure (Figure 34).

Eggs from adult medaka fish exposed to 300 mM alcohol 2 h daily for 30 days, were collected each day and separated into fertilized and unfertilized and their count were recorded daily (Figure 35). At the end of the 30 days, fish were sacrificed and the ovary and liver of each fish were harvested for paraffin embedding and tissue sectioning, and biochemical analysis (Figures 36-46). Eggs from the 28, 29 and 30 day of the experiment were maintained for circulatory status and trabecular cartilage detection in the offspring as a FASD like character (Figures 47-50).
Figure 34: Blood alcohol concentration (BAC) in the adult fish. Adult fish were exposed to 300 mM alcohol in 400 ml hatching solution using 1-liter filter system bottles for 2 hours. Fish were then sacrificed and the blood was collected from the heart of the fish. BAC was determined following Rimers et al., (2004), n = 4.
1.1 Effect of chronic alcohol exposure on egg production and fertilization of reproductively active female fish chronically exposed to alcohol.

In this aim we investigated the effect of chronic high blood alcohol level on the fecundity of reproductively active medaka fish. Fertilized eggs from females of alcohol treated groups were significantly decreased in comparison with controls while unfertilized egg numbers did not show significant change between alcohol treated groups and controls.

Figure 35: Effect of chronic high BAC on fecundity of medaka fish. Fertilized eggs were significantly reduced in number in female only treated and in male and female treated groups in comparison to controls but both alcohol-treated groups were not significantly different from each other. Unfertilized eggs even though showed a tendency to increase in alcohol treated male and female group but it did not show any significant difference from female only treated group nor controls; n = 4-8 fish / group, one-way ANOVA p < 0.05.
1.1 Determine histological and biochemical effects of chronic alcohol exposure on adult fish ovary and liver.

We have analyzed the ovarian histology of reproductively active medaka chronically exposed to alcohol. After 30 days of chronic alcohol exposure medaka fish were sacrificed and their ovaries and livers were collected and divided for histological sections as well as biochemical analysis. It was observed that medaka ovary consists of both pre-vitellogenic and post-vitellogenic oocytes (Figures 36 and 37). We counted the number of pre-vitellogenic and post-vitellogenic oocytes in both control ovaries and chronically alcohol treated ovaries (Figure 38 A). We observed that the number of pre-vitellogenic oocytes were significantly higher in alcohol treated female ovaries in comparison with controls. However the number of post-vitellogenic oocytes was significantly higher in control fish compared to alcohol treated fish (Figure 38 B).

Figure 36: H&E stained section of medaka female control ovary showing both mature and early stage ova. Ova were characterized by size as well as the presence of yolk granules in the ooplasm.
Figure 37: H&E stained section of medaka female ovary chronically treated with alcohol. Fibrosis of the ovarian stroma below the germinal epithelium and to the center can be seen. Also the sections shows a decreased number of post-vitellogenic and mature oocyte in favor of the number of pre-vitellogenic oocyte, which is opposite to what can be seen from the control ovary.
Figure 38: The effect of chronic alcohol exposure on oocyte population of adult female ovary. We counted the number of pre-vitellogenic oocyte (A) and post-vitellogenic oocyte (B). The number of pre-vitellogenic oocytes was significantly higher in alcohol treated female ovaries in comparison with controls. However the number of post-vitellogenic oocytes was significantly higher in control fish compared to alcohol treated fish; n=4, Student T test $p < 0.05$. 
After observing the significant changes in ovarian histology induced by alcohol exposure, we have further analyzed several biochemical parameters related to alcohol metabolism, and oxidative stress that could be generated by alcohol exposure. We investigated the effect of chronic alcohol exposure on mRNA expression of adh5, aldh1a2, aldh2, adh8, and aldh9 as well as oxidative stress enzymes catalase, SOD and GST in the ovaries of treated females. Alcohol metabolizing enzymes adh5, aldh1a2, and aldh2, are the main alcohol-metabolizing enzyme in medaka, and adh8, and aldh9, are ancestor enzymes. Our results showed no significant change in the mRNA concentration of adh5, adh8 and aldh1a2 in the ovaries of alcohol treated group compared with controls. Significant reduction was seen in the mRNA concentration of aldh2 while significant elevation was seen in the mRNA concentration of aldh9 in the ovaries of alcohol treated females in comparison with controls (Figures 39-40). For the mRNA concentration of the oxidative stress enzymes GST showed significant reduction, while SOD showed significant elevation and catalase was not altered in the ovaries of alcohol treated group in comparison with controls (Figure 41).
Figure 39: Effects of chronic alcohol exposure on adh 5(A), adh8 (B), mRNA expression in the ovary of adult female medaka chronically treated with alcohol. No significantly different changes in the mRNA expression of these 2 enzymes were observed between control and alcohol treated group; n = 4-8, student t test \( p < 0.05 \).
Figure 40: Effects of chronic alcohol exposure on aldh 1a2 (A), aldh 2 (B), and aldh 9 (C) mRNA expressions in the ovary of adult female medaka chronically treated with alcohol. No significantly different change in the mRNA expression of aldh 1a2 was detected between alcohol treated and control females. On the other hand aldh 2, and aldh 9 mRNA expression was significantly reduced in the ovaries of alcohol treated females relative to the ovaries of control females; n = 4-8, student t test p < 0.05.
Figure 41: Effects of chronic alcohol exposure on antioxidant enzyme catalase (A), GST (B), and SOD (C) mRNA expression in the ovary of adult female medaka chronically treated with alcohol. No significantly different changes in the mRNA expression were observed between control and alcohol treated group for catalase while SOD was significantly increased and GST was significantly decreased when compared to control group; n = 6-8, student t test p < 0.05.
Figure 42: H&E stained section of control medaka female liver showing normal liver architecture with the arrows referring to the central veins (A). Higher magnification showing details (B). H&E section of medaka female liver chronically treated with alcohol showing fibrosis (stained with eosin appearing orange reddish in color). Dilated central veins and loss of architecture can also be seen in the section (C). Higher magnification showing details (D).
Our data indicated that chronic alcohol exposure had significant impact on the histological structure of the liver. We have also observed disruption of medaka fecundity after chronic alcohol exposure. Vitellogenin is a protein precursor synthesized in the liver and provides the major egg yolk proteins that are a source of nutrients during early development of oocytes in both vertebrates and invertebrates. We therefore measured some biochemical components, of the liver of female medaka chronically exposed to alcohol, that are related to egg production as well as alcohol metabolism and oxidative stress.

![Vitellogenin](image)

Figure 4: Effect of chronic alcohol exposure on medaka liver vitellogenin mRNA expression. Significant reduction in the mRNA expression of vitellogenin in the alcohol treated female liver in comparison to controls was seen. This is corresponding to the decrease in number of mature eggs in alcohol treated group, n = 4-8, student t test p < 0.05.
Figure 44: Effects of chronic alcohol exposure on \textit{adh 5} (A), \textit{adh 8} (B), mRNA expression in the liver of adult female medaka chronically treated with alcohol. Both enzymes showed significant reduction in their mRNA expression in alcohol treated group in comparison with control; \( n = 4-8 \), student t test \( p < 0.05 \).
Figure 45: Effects of chronic alcohol exposure on *aldh1a2* (A), *aldh2* (B), and *aldh9* (C), mRNA expression in the liver of adult female medaka chronically treated with alcohol. Significantly different decrease in the mRNA expression of these enzymes between control and alcohol treated group were detected indicative of oxidative stress; n = 4-8, student t test p < 0.05.
Figure 46: Effects of chronic alcohol exposure on antioxidant enzyme \textit{catalase} (A), \textit{SOD} (B), and \textit{GST} (C), mRNA expression in the liver of adult female medaka chronically treated with alcohol. Significantly different decreases in the mRNA expression of these enzymes between control and alcohol treated group indicative of oxidative stress were detected; \( n = 4-8 \), student t test \( p < 0.05 \).
1.2 Measure circulation status, and trabecular cartilage development in the offspring.

Our results showed that there is disruption of the fecundity of alcohol treated medaka females with decreased in the numbers of fertilized eggs. We further investigated the possibility of alcohol inducing epigenetic changes in the parent fish that will be transmitted to the offspring causing FASD like deformities. We examined the circulatory status and neurocranial cartilage deformities as phenotypic markers of alcohol toxicity.

Figure 47: Percentage of circulating embryos on 6 dpf from the last 3 days of chronic parent exposure experiment (parents Male & Female exposed to alcohol 300 mM 2h/day for 30 successive days) in control and treatment groups. Both control and treatment group had a 100% circulating embryos on 6 dpf, n =16.
Figure 48: Cartilage staining with Alcian blue of 7 day medaka embryos from the last 3 days of chronic parent exposure experiment (parents Male & Female exposed to alcohol 300mM 2h/day for 30 successive days) control group (A) and parent chronically exposed to alcohol (B). They both have intact trabecular cartilage of comparable length n = 16.

Figure 49: Presence of trabecular cartilage in treatment groups. Both control (C) and Parent treatment (T) group has a 100% presence of trabecular cartilage with no defects detected in any group n = 8-12.
Figure 50: Effect of chronic alcohol exposure of parent madaka on TC and NC length on 7 dpf embryos from the last 3 days of chronic parent exposure (parents Male & Female exposed to alcohol 300mM 2h/day for 30 successive days) in control and treatment groups. No significant difference was detected between the cartilage length in both groups; n= 6-8 student T test P<0.05.
CHAPTER 5
DISCUSSION

5.1 PG as a potential preventive agent of FASD

One of the potential problems in studying alcohol teratogenesis is finding a suitable animal model for experimental use. Every animal model is different and it is also very difficult to find a single animal model that can mimic the human conditions (Faut et al., 2009, Haron et al. 2013). For many years now Japanese medaka embryogenesis has been used in our laboratory as an animal model to study teratogenic effects of alcohol (Dasmahapatra et al., 2005, Wang et al., 2006, 2007a, b, Hu et al., 2008, 2009, Wu et al., 2008, 2011, Haron et al., 2013). Others have subsequently used this model and it is considered to be a unique fish model for studying alcohol teratogenesis (Oxendine et al., 2006a, 2006b). Our previous studies have shown that alcohol was able to induce teratogenic features in medaka embryos that are considered analogous to human FASD phenotypes (Haron et al., 2012). Alcohol-induced teratogenic effects in fertilized medaka embryos include delay in initiation of vessel circulation, formation of thrombi in many regions of the body including the brain, microcephaly (small head size), malformed neurocranial and trabecular cartilages, and alteration in oxidative stress enzymes (Wu et al., 2008, 2011, Haron et al., 2012).

One of the aims of this study was to develop a drug from a natural product that is otherwise harmless when consumed during pregnancy. Many reports in *Chinese Materia Medica*
have been published, “Ben Chao Gang Mu” in 1590-1596 AD, described the use of combinations of certain plant extracts including Panax ginseng, in the treatment of alcohol toxicity (Tomczyk et al., 2012). A recent report showed that black ginseng is able to attenuate alcohol teratogenesis in mouse embryos in vitro (Lee et al., 2009). This prompted us to evaluate the efficacy of ginseng as a preventive agent of FASD by blocking alcohol-induced oxidative stress (Figure 1). We utilized our Japanese medaka embryogenesis model of FASD for the screening of potential drugs that can prevent or attenuate FASD (Haron et al., 2013).

We calculated the lethal concentration of PG on medaka embryogenesis to be 355.5 ±1.12 µg/ml in A group and 679.6 ± 1.6 µg/ml in B group (Figure 2). This shows that B group embryos were tolerant to higher concentrations of PG than A group embryos by almost double the dose (355.5 µg/ml in A group v/s 679.6 µg/ml in B group). Embryo survivability was severely affected with 100% mortality at concentrations > 500 µg/ml for A group embryos, and >750 µg/ml for B group embryos. Initiation of vessel circulation, and hatching efficiency were also severely affected by high PG concentrations especially at 0-2 dpf (Figure 3). These effects could be due to inability of the embryo at this early stage (0-2 dpf) to efficiently metabolize PG. From these initial experimental data we have set up the maximum limit of PG concentration to be 200µg/ml in all other experiments.

In this study we have used sub-lethal concentrations of PG (50-200 µg/ml) as an antagonist of alcohol toxicity. In A group embryos, supplementation of PG at 50-100 µg/ml with A 300 mM was unable to protect the embryos against alcohol toxicity. The mortality remained at the same level as in A 300 mM only group. This may be due to inability of the embryo, at this early developmental stage, to metabolize PG to more active metabolites that are able to prevent
alcohol toxicity and improve embryo survivability. Adding PG 200 μg/ml with A 300 was toxic for the A group embryos at the end of 48h exposure time. This is indicating a synergistic toxic effect between alcohol and PG at this early stage of development (Figure 4). On the other hand, in B group, when PG 50-100 μg/ml was added simultaneously with A 300 mM embryo survivability was not significantly different from the corresponding controls. However, when PG concentration increased to 200 μg/ml, significant toxicity was observed and embryo survivability was reduced to 60 % (Figure 4). These results indicate that PG 50-200 μg/ml is unable to improve Japanese medaka embryos survivability from alcohol toxicity at blastula stages, but PG 50-100 μg/ml can improve alcohol exposed embryo survivability in neurula stages. This could be explained by the improved ability of the embryos at later stage (1-3 dpf) to metabolize PG to more active metabolites. Izzo and Ernest (2001) reviewed the literature to determine the possible interactions between the seven top-selling herbal medicines (ginkgo, St John's wort, ginseng, garlic, echinacea, saw palmetto and kava) and prescribed drugs. They reported that *Panax ginseng* lowers blood concentrations of alcohol. This decrease in blood concentrations of alcohol could be through enhancing alcohol metabolism, which could in turn explain why higher concentrations of ginseng (PG 200 μg/ml) in A 300 + PG 200 group in blastula stage caused enhanced mortality when compared with A 300 and A 300 + PG 50 groups. PG could be enhancing alcohol metabolism leading to higher levels of oxidative stress, which could have caused the enhanced mortality in this group. Further investigations are needed to explain the synergistic mechanism between alcohol and ginseng by which it is causing death of the medaka embryos.

The onset of vessel circulation is generally initiated at 50 hpf (Iwamatsu stage 25) in
normal embryogenesis (Iwamatsu, 2004). Vessel circulation is an indication of the successful progression of morphogenesis (Solomon and Weis, 1979). Embryos that failed to initiate vessel circulation in ovo generally failed to hatch spontaneously (Solomon and Weis, 1979, Taneda et al., 2010). Japanese medaka embryos exposed to only PG 50-200 μg/ml containing media, were able to initiate vessel circulation like the controls at 50 hpf. Exposing embryos to A 300 mM at 1-3 dpf led to delayed onset of circulation when compared to controls, however these embryos were still able to initiate vessel circulation by 6 dpf. Supplementation of PG at 200 μg/ml to embryos at 1-3 dpf simultaneously with A 300 mM, significantly reduced initiation of vessel circulation as compared to controls and A 300 only groups. This also indicates a synergistic toxic effect from both alcohol and high concentration of PG on medaka embryos. Vessel circulation was at comparable levels with controls and A 300 groups, when PG at 50-100 μg/ml was supplemented at 1-3 dpf simultaneously with A 300 mM (Figures 5 and 6). The circulation and hatching results correspond to what was seen in survivability data. This could be explained by inability of medaka embryos at the very early stages of development to synthesis enzymes needed to metabolize PG or PG could be causing enhanced alcohol metabolism generating more oxidative stress or both (Izzo and Ernest 2001). Alternatively PG could be targeting some other as yet unidentified mechanism in the medaka embryos and this mechanism is enhanced by simultaneous alcohol exposure leading to death of the embryo (Haron et al., 2013).

TC development in medaka embryos is a potential target of alcohol toxicity (Hu et al., 2009). Medaka embryos exposed to A 300 mM 0-2 or 1-3 dpf had microcephaly (small head) with deformed TC (Hu et al., 2009). Therefore we tested whether PG would have a protective effect against alcohol toxicity as manifested by induced TC deformity in B group embryos. TC is
developed by 5-6 dpf in normal embryos (Iwamatsu, 2004). Alcian blue staining of B group embryos at 7 dpf showed that adding PG 50-200 μg/ml at 1-3 dpf simultaneously with A 300 mM protected the embryos against TC deformity induced by alcohol (Figure 7). Effect of PG on TC linear length when simultaneously supplemented with alcohol at 1-3 dpf was comparable to controls, however; PG was not able to reverse the reduction in linear length of neurocranium due to alcohol toxicity (Figure 8). This could be explained by the improved ability of the embryo at this stage (1-3 dpf) to metabolize PG yielding active metabolites that are able to block oxidative stress generated from alcohol metabolism and partially protect the embryo (Lee et al., 2009).

We tested the possibility that PG is affecting alcohol permeability through the chorion of Japanese medaka embryos and changing embryonic alcohol concentration. PG supplementation did not alter alcohol concentration in the embryos (33% in A 300 vs. 28% in A 300 + PG 100 of the media concentration). This suggests that PG has some other as yet unidentified mechanism by which it is modulating the alcohol effect on medaka embryogenesis (Figure 9).

The reduction in *adh8* and *GST* mRNA expression in alcohol treated embryos in comparison to controls suggests that the toxic effects of alcohol in Japanese medaka embryogenesis are mediated by the generation of oxidative stress (Wu et al., 2011, Haron et al., 2012, Bleoanca et al., 2013, Sid et al., 2013). Blocking oxidative stress could be one of the possible mechanisms by which PG is protecting against alcohol toxicity. We investigated the methanolic root extract of PG (also known as black ginseng), which contains major ginsenosides known for their antioxidant activities (Lin et al., 2013, Sun and Chen, 2011), for its possible protective effect against alcohol-induced oxidative stress in medaka embryogenesis (Karmazyn et al., 2011). We focused on mRNAs of the alcohol metabolizing enzymes (*adh5, adh8, aldh2,*
and aldh9) and the enzymes that are related to oxidative stress (catalase, GR, GST). Among these enzymes adh8, aldh9, and GST which are developmentally regulated (Dasmahapatra et al., 2005, Wang et al., 2007, Wu et al., 2011). We observed that alcohol alone significantly reduced the mRNA content of adh8 and GST in comparison to the controls, while mRNA expression of adh5, aldh2, aldh9a, catalase, and GR remained unaltered. Adding PG 100 μg/ml simultaneously with A 300 produced the same results as A 300 alone (Figures 10, 11, and 12). This indicates that PG was unable to reverse alcohol-induced effects on alcohol metabolizing enzymes and on oxidative stress enzymes gene expression. Further investigations are needed to verify the possibility of ginsenosides working as antioxidants by scavenging the free radicals.

We analyzed the exposure media for ginsenoside concentrations. HPLC analysis showed the separation of the different ginsenosides present in the different types of media that were used in this study in comparison to a standard ginseng root extract. There were almost no differences between ginsenoside concentrations in the solutions with and without alcohol in the media (Figures 13 and 14) and (Table 1). This suggests that the biphasic nature of PG action, which was seen at the different developmental stages, is related to the total concentration of PG in the media. This is because adding alcohol to the different PG concentrations did not have any modulating effect on the concentration and bioavailability of the ginsenosides present in the PG crude extract that we used.

5.2 Effects of antioxidant

PG supplementation did not change the alcohol-induced effects on alcohol metabolizing enzymes and oxidative stress enzymes mRNA expression. We investigated the possibility that
the ginsenosides in the PG root extract could be working through scavenging reactive oxygen species or reactivation and augmentation of endogenous antioxidant compounds. In zebrafish, alcohol dependent toxicity is partially attenuated by antioxidants (Reimers et al., 2006). We investigated known model antioxidant compounds, e.g. N-acetyl cysteine and ascorbic acid, for their protective effect against alcohol toxicity (Mullins and Vitkovitsky 2011, Setshedhi et al., 2011, McDonough, 2003, Harikrishnan et al., 2013). N-acetyl cysteine and ascorbic acid were able to protect medaka embryos against disruption of vessel circulation seen in embryos exposed at 1-3 dpf to A 300 alone (Figure 15). Linear length measurements showed that all groups except for A 300 (which was significantly shorter) had a linear length comparable to controls for TC (Figure 16 and 17 A). For the NC cartilage A 300 and A 300 + PG 50 μg/ml were the only treatment groups that were significantly different from controls. All other treatment groups including the A 300 + antioxidant were comparable in length with controls (Figure 17 B). This suggests that blocking oxidative stress by antioxidants can protect medaka embryos against alcohol-induced teratogenesis (Goodlett et al., 2005). Similar results were reported by Heaton et al., (2006) who found that supplying antioxidant compounds e.g. vitamin E was able to protect rat embryos against neural manifestations of alcohol toxicity.

Our data showed important differences between PG and antioxidants regarding their protective effect against alcohol toxicity. Antioxidants model were able to provide effective protection against alcohol toxicity by significantly improving TC and NC cartilage linear lengths with having no adverse effect on circulation. PG was only able to protect TC linear length with having adverse effects on circulation at PG 200 μg/ml. This paradoxical effect of PG could be due to two counteracting mechanisms. The first mechanism is working through PG causing
enhanced alcohol metabolism leading to more oxidative stress in the embryo. The second mechanism is working through antioxidant activity of ginsenosides. The net result of these two mechanisms is a partial protective effect of ginseng that is less effective than the antioxidant model, which works primarily through scavenging reactive oxygen radicals. Further investigations are needed to verify these mechanisms.

5.3 Alcohol induced neural cell alteration in medaka cerebellum

The most serious characteristic of FASD is the neurological damage that results from prenatal exposure to alcohol. This can cause serious neuropsychological alterations that would affect not only FASD individuals but also the whole society. Alcohol-induced neurological damage is invisible, making it hard to diagnose and hard to treat (Haron et al., 2012). We investigated the possibility of inducing neural cell alterations characteristic of FASD in Japanese medaka as a model and the possible protective effect of PG.

The cerebellum is one of the most vulnerable sites to growth restriction and neuronal depletion induced by alcohol exposure during the brain growth spurt (Goodlett et al., 1991). The cerebellar cortex has three distinctive layers of cells, the inner most granular layer, the outer most molecular layer and the Purkinje cell layer in between (White and Sillitoe, 2013). The Purkinje cell layer contains the cell bodies of the Purkinje cells, which are a class of GABAergic neurons and are among the largest neurons in the human brain with an intricate dendritic net (Rochefort et al., 2013). Different animal studies have shown that low blood-alcohol levels could produce both brain damage and behavioral abnormalities associated with permanent decreases in the Purkinje and granule cell numbers in the cerebellum (Napper and West, 1995).
Purkinje cells are responsible for all motor coordination from the cerebellar cortex (Rochefort et al., 2013). Purkinje cell atrophy shortly after birth, or failure to develop in utero, or die off before birth can lead to symptoms such as ataxia, intention tremors, hyperreactivity, stiff or high-stepping gait, lack of awareness of foot position (walking with a foot knuckled over), and inability to determine space and distance (Purves et al., 2008, Kirsch et al., 2012). Recent studies have suggested that Purkinje cells play an important role in our brains cognitive ability and information processing (Forrest et al., 2012).

In humans, Purkinje cells can be harmed by multiple causes: toxic exposure, e.g. to alcohol or lithium, neurodegenerative diseases, and autoimmune diseases (Xia et al., 2013). Histological sections of cerebella of prenatally alcohol exposed medaka showed apparent reduction in the number and size of cells in the Purkinje cell layer in comparison to controls (Figures 18 and 19), and adding PG 50 μg/ml simultaneously with alcohol at this early stage of embryonic development had a significant protective effect against alcohol toxicity to Purkinje cells (Figures 20 and 21). Prenatal alcohol exposure showed significant toxicity manifested by decreased number and size of the Purkinje cells. Adding PG to alcohol exposure protected the cells (Figures 22 and 23). This effect could be mediated by blocking oxidative stress or by neuro-stimulatory mechanisms of PG or both (Reddy et al., 2013, Ramezani, 2012, Lee et al., 2009). Further investigation is needed to establish the exact mechanism (Lee et al., 2009, Heaton et al., 2006, Goodlett et al., 1997, Samson, Diaz, 1981). Some of these finding have also been established in other models e.g., mouse. However establishing these findings in medaka is of particular importance because of the advantages of having a simpler nervous system, simpler genome, short life cycle, and low maintenance cost. These advantages of medaka over
mammalian models make it appealing for researchers to use medaka as an alternative animal model if it can be proven to be an effective mimic of the human system.

We also investigated neuroligin, which is a cell adhesion protein on the postsynaptic membrane that mediates the formation and reinforcement of synapses between neurons (Xu et al., 2012, Scheiffele et al., 2000). Mutations of neuroligin have been implicated in autism and other cognitive dysfunction syndromes (Bottos et al., 2011). First we investigated the expression of \( nlgn \ 1, 2a \) and \( 2b \), and \( 3a \) and \( 3b \) during normal fetal development of medaka embryos. We observed that for \( nlgn \ 1 \) the maximum expression was at 0 dpf, which suggests that it could be coming from the mother (Figure 24). The maximum expression of \( nlgn \ 2a \) was at 6 dpf, which suggest developmental regulation (Figure 26). For \( nlgn \ 2b \) maximum expression was detected at two-time points 0 dpf suggesting that it is coming from the mother, and hatchling stage suggesting developmental regulation as well (Figure 28). For \( nlgn \ 3a \) and \( 3b \) maximum expression was seen in hatchlings (Figures 30 and 32). These findings suggest a developmental regulation of the different \( nlgn \) genes during medaka embryogenesis. Treating B group embryos with A 300, PG 50, and A 300 + PG 50 along with controls and measuring the mRNA expression of the different \( nlgn \) genes on 6 dpf did not result in any significant changes in any of the \( nlgn \) genes expressions during medaka embryogenesis. These results showed that alcohol exposure during embryogenesis did not have any effect on \( nlgn \) gene mRNA expression. This suggests that the cognitive changes that may be seen in FASD individuals are not the result of changes in the \( nlgn \) gene expression (Figures 25, 27, 29, 31 and 33).

5.4 Effects of chronic alcohol exposure on female medaka fecundity
In this part of the study we investigated the hypothesis of trans-generational epigenetic inheritance of traits related to alcohol toxicity from exposed parents. We hypothesized that some epigenetic changes can occur due to chronic alcohol exposure, and that these changes are transmitted to the offspring leading to some of the neurobehavioral and morphological deformities characteristic of FASD.

Both male and female medaka exposed to A 300 for 2 hours, exhibited similar blood alcohol concentrations (Figure 34). In this study we have concentrated on the effects of chronic alcohol exposure on the fecundity of female medaka fish. However we had exposed male medaka fish to test for the effect of chronic exposure on their ability to fertilize female fish and the inheritance of FASD traits in the offspring. Our results showed that the numbers of fertilized eggs in relation to daily production and body weight in both female only and male and female treated groups were significantly decreased from the control group (Figure 35 A). The number of fertilized eggs was significantly reduced compared with controls in both the male and female treated and the female only treated group. However, there was no significant difference between the two treated groups indicating that the primary effect was due to treatment of the female. On the other hand the number of unfertilized eggs was not significantly different from controls in all treatment groups (Figure 35 B). Analyzing histological sections from the ovaries of alcohol treated females showed what seemed to be oocyte arrest in the early stages of ova development when compared to histological sections of control ovaries, which showed more mature stage ova (Figures 36 and 37). By counting the number of mature eggs in histological sections of ovaries of alcohol treated and control medaka females we observed that the number of post-vitellogenic and mature oocytes was significantly lower in alcohol treated female ovaries than in controls (Figure
Moreover the number of early stage oocytes (pre-vitellogenic) was significantly higher in alcohol treated compared with controls (Figure 38 A).

Vitellogenin, which is a precursor protein important for egg development and maturation, is secreted from the liver under the influence of estrogen and then goes to the ovary to be deposited in the pre-vitellogenic oocytes allowing them to grow to mature oocytes. We measured the mRNA expression of vitellogenin in the liver and our data showed significantly decreased expression of vitellogenin mRNA in the liver of alcohol treated females (Figure 43). This observation can be correlated to the previous observations of oocyte arrest at early stages in the ovaries of alcohol treated females and the decreased number of fertilized eggs (Figures 35, 38, and 43). Decreased vitellogenin expression in the livers of alcohol treated females led to a decrease in the vitellogenin available in the ovaries. This decreased the ability of the oocytes to grow to mature stage and be fertilized.

The H&E histological section of ovaries from the alcohol treated females showed fibrous tissue deposition in the cortical stroma below the germinal layer and inside the ovarian stroma in comparison to controls. Fibrosis is an indication that tissue repair has occurred (Ghosh et al., 2013) (Figures 36 and 37). This observation is indicative of tissue injury, which may have been caused by oxidative stress from alcohol toxicity. Therefore we further investigated mRNA expression of alcohol-metabolizing enzymes and oxidative stress enzymes in the ovaries of alcohol treated females and compared these to controls. Our data showed that adh5 and adh8 mRNA expression in the ovaries of the alcohol treated group were not significantly different from the control group (Figure 39). Meanwhile aldh2 was significantly lower in the alcohol treated groups than controls while aldh9 was significantly
higher and aldha2 remained unaltered (Figure 40). While catalase mRNA expression in alcohol treated female ovaries was similar to controls, GST mRNA expression showed significant reduction, and SOD mRNA expression showed significant increase in the alcohol treated group compared with controls (Figure 41). These data are indicative of ovarian oxidative stress in the alcohol treated females in comparison to control females. This can be correlated to the tissue injury and fibrosis, and decreased fertility that was seen in the ovaries of this group (Faut et al., 2009, Jagielska et al., 2012).

The liver is the main organ for detoxification of alcohol and production of vitellogenin which is an essential protein for egg maturation (Zhang et al., 2011). We investigated the effect of chronic alcohol exposure on the livers of the alcohol treated females. H&E stained histological samples of the adult female liver chronically exposed to alcohol demonstrated some alterations from controls (Figure 42). The alcohol treated female liver showed fibrous deposition around the hepatocytes and some central vein dilation and loss of texture when compared to controls. This is indicative of tissue injury followed by fibrosis (Ghosh et al., 2013) (Figure 42 C and D).

We further investigated the expression pattern of alcohol metabolizing enzymes and oxidative stress enzymes in liver samples from alcohol treated and control females. We found that adh5 and adh8 mRNA in alcohol treated female livers was significantly lower than controls (Figure 44). Liver aldha2, aldha2, and aldha9 were also significantly decreased in alcohol treated females compared to controls (Figure 45). Furthermore, mRNA expression patterns of the oxidative stress enzymes catalase, GST, and SOD in alcohol treated female livers were also significantly lower than for the control group (Figure 46). These data
correlate with the histological findings as indicative of oxidative stress injury to the liver and ovary. The fibrosis induced in the liver caused central veins to dilate and to lose architecture. This implies possible loss of liver function manifested by decreased mRNA expression of alcohol-metabolizing enzymes, oxidative stress enzymes, and vitellogenin. This loss of function decreased the fecundity of the female medaka (Arteel et al., 1996, and Norberg et al., 2003).

5.5 Effects of chronic alcohol exposure of the parent medaka fish on their offspring

Offspring from medaka parents, chronically treated with alcohol for 30 days, were tested for FASD like features. Eggs collected from alcohol treated females only, alcohol treated male and female, and control females on 28, 29 and 30 days of the exposure were cultured until hatching under normal conditions. Circulatory status (Figure 47) and trabecular cartilage (Figure 48, 49 and 50) of the offspring were assessed. Our results showed that none of these parameters were altered in the offspring of the alcohol treated parents in comparison to controls. These data did not support our hypothesis that some epigenetic alterations may have occurred in the alcohol-exposed parents that could be transmitted to their offspring and cause FASD like features. In this study we were unable to demonstrate any significant FASD features in the offspring of parents chronically treated with alcohol. Further investigation is needed as it is still possible that FASD features may be epigenetically transmitted that may not be expressed until later generations.
CHAPTER 6
CONCLUSION

When we started this study our goal was to find a treatment for FASD from a natural product. Using Japanese medaka as our animal model for FASD we tested the ability of Asian ginseng to ameliorate or prevent FASD like features. PG was able to modulate alcohol induced teratogenesis and toxicity in Japanese medaka embryogenesis in a biphasic manner. However while PG potentiated alcohol toxicity and enhanced embryo mortality in the blastula stage, it was able to protect the medaka embryo from multiple features of alcohol toxicity in the neurula stage. Comparing the PG protective effect with that of model antioxidants showed that the model antioxidants were more effective in protecting medaka embryo against prenatal alcohol toxicity. From our results and other literature reviewed in this study PG and antioxidants could provide a good base for a drug being developed to prevent or ameliorate FASD in human pregnancy.

We have also succeeded in developing a neural model of FASD in medaka fish embryonically treated with alcohol, and we have shown that PG has a protective effect against some of the alcohol induced neural alterations at early embryonic stages. This suggests that medaka can be an effective alternative to mammalian models for studying FASD due the advantages mentioned earlier in this study. Further studies are needed to verify the possibility and the recommended doses of ginseng that can be used during human pregnancy.
We have also developed an adult fish model of chronic alcohol exposure. Chronic alcohol exposure of medaka females caused significant reduction in their fecundity. Even though we have not been able to verify all the possible mechanisms in this study, our data have shown significant histological and biochemical alterations in the ovaries and livers of female medaka caused by chronic alcohol exposure. While these alterations did not cause any apparent FASD like features in the offspring, they did show that this model could be used for further exploration of the molecular targets of alcohol that can cause indirect embryonic toxicity features in the offspring. The result of disrupted fecundity seen in female medaka after chronic alcohol exposure may help to shed some light on other alcohol toxicity symptoms e.g. stillbirth and preterm birth seen in human pregnancies, which could be the result of disrupted egg development and fertilization in women chronically exposed to alcohol.
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Table 2 Primers used in qPCR amplifications of the alcohol metabolizing enzyme and antioxidant enzyme mRNAs of *Oryzias latipes* embryos.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense (5’-3’)</th>
<th>Antisense (5’-3’)</th>
<th>Product size (bp)</th>
<th>Oxidative stress/ alcohol metabolism</th>
<th>Ensembl number/GenBank accession</th>
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<tr>
<td>Catalase</td>
<td>gcggtacaacgcgcagatgaag</td>
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<td>Oxidative stress</td>
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<td>GST</td>
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<td>ggcctcaacatgcggttgttgg</td>
<td>241</td>
<td>Oxidative stress</td>
<td>GenBank X95200</td>
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<td>cattgactctccatgcgttgtgtg</td>
<td>165</td>
<td>Oxidative stress</td>
<td>ENSORLT00000000771</td>
</tr>
<tr>
<td>SOD</td>
<td>gcacactctgtacgcgctcactc</td>
<td>ttaccaagtttccgggtgttgg</td>
<td>276</td>
<td>Oxidative stress</td>
<td>ENSORLG000000019434</td>
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<td>Adh5</td>
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<td>gcacacgacttttcgagccggctcagccc</td>
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<td>gtcacacgatgcttgcagccc</td>
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<td>Alcohol metabolism</td>
<td>GenBank AY682722</td>
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<td>Aldh1a2</td>
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<tr>
<td>EF-α1</td>
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<td>300</td>
<td>Internal standard</td>
<td>GenBank NM_001104662</td>
<td></td>
</tr>
</tbody>
</table>

**100X stock of hatching solution:**

1. 9.945 g of NaCL.
2. 198mg of KCL
3. 1.48g of MgSO₄
4. 619mg of CaSO₄
5. Dilute to 1X before use.

**50X stock of TAE solution for gel electrophoresis:**

1. 242g Tris (pH 8)
2. 57.1 ml Glacial acetic acid
3. 100 ml of 500mM EDTA
4. Add Nano pure water up to 1 Litre.
5. Dilute to 1 X before use.

**1% agarose gel:**

1. Add 0.8 g agarose powder to 40ml 1X TAE solution.
2. Heat for 45 seconds in the microwave until it dissolves.
3. Add 10μl ethidium bromide to the mixture and shake gently.
4. Pour gel mixture in the casting tray, and insert comb.
5. Leave to solidify in room temperature.

**RNA extraction:**

1. Put 8 embryo in 1.5ml eppendroff tube
2. Add 100 μl of Trizol reagent.
3. Homogenize them thoroughly.

4. Add another 400 µl of Trizol reagent for a total of 500 µl (you can store at -80°C for future use or proceed).

5. Add 100 µl of chloroform and leave in room temperature for 5 minutes.

6. Vortex and spin at 12000 rpm for 20 minutes.

7. After spinning the tube will have 3 phases red at the bottom, white in the middle and clear supernatant at the top. Take the supernatant carefully without touching the white fluffy layer.

8. Add 500 µl of 2-propanoland and leave in room for 10 minutes or you can store at 4°C overnight.

9. Spin at 12000 rpm for 20 minutes at 4°C.

10. Remove the supernatant carefully without losing the white pellet.

11. Add 1 ml of 75% ethanol and vortex to loosen the pellet.

12. Repeat step 9.

13. Remove the supernatant carefully and allow drying for around 1-2 hours.


15. You can store at 80°C for future use or proceed.

16. Removal of DNA for a total of 50 µl:

   a) Add 10 µl of 5X transcription buffer.

   b) 0.5 µl of 5mM MnCl₂.

   c) 1 µl of RNasin.

   d) 1 µl of RQ1 DNase.
e) 7.5 µl of Nuclease free water.

f) Heat at 37 for 30 minutes.

g) Add 500 µl Trizol.

h) Then repeat from step 5 until 14.

According to the guidelines produced under the direction of the Scientific Advisory Committee of the Royal College of Obstetricians and Gynaecologists as an educational aid to obstetricians and gynaecologists:

1 unit of alcohol approximately equals 8 gms of absolute alcohol which is equivalent to:

- ½ pint of ordinary strength beer, lager, cider
- ¼ pint of strong beer or lager
- 1 small glass of wine
- 1 single measure of spirits
- 1 small glass of sherry
VITA

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