Exploring a Fish Model of Developmental Neurotoxicity Using the Japanese Medaka Fish (*Oryzias latipes*)

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ABSTRACT

SHARON L. OXENDINE: Exploring a fish model of developmental neurotoxicity using the Japanese medaka fish (*Oryzias latipes*)

Ethanol exposure induces profound disruptions in developing organisms, but the period(s) of maximal susceptibility for these effects are not clearly defined. The overall hypothesis of this dissertation was that the timing of developmental ethanol exposure would influence manifestations of ethanol toxicity in medaka. Hatching success and morphometric measures of medaka growth and development were used to assess the dose-response for developmental ethanol toxicity. Specifically, medaka embryos were exposed to a range of sublethal ethanol concentrations (0.1, 0.5, ot 1.0 % ≡ 17.2, 86, or 172 mM) during early, middle or late development in order to address temporal variations in sensitivity. Ethanol-induced decreases in total body length were found to be most severe with increasing ethanol concentrations that occurred either early or late in embryonic development, whereas head width was consistently decreased at all ethanol concentrations, regardless of developmental timing. These effects were accompanied by dose-related decreases in hatching success which were more pronounced with ethanol exposures that occurred early in development. Subsequent analyses of time-dependent changes in brain growth and caspase-3/7 activation were used to address the neurodevelopmental consequences of ethanol exposure. In these studies, medaka embryos were exposed to 1% ethanol during early, middle or late development and evaluated for treatment-related changes in brain volume and caspase-3/7 activation on day 9 postfertilization. Interestingly, the pattern of sensitivity observed for ethanol-induced decreases in total brain volume was similar to that seen with ethanol-induced decreases in somatic growth; embryos treated either early or late in development were most severely affected. Treatment-related decreases in caspase-3/7 activity were independent of

developmental timing. In general, these data show that the sensitivity to developmental ethanol exposure varies according to both the timing of exposure and the endpoint used to assess toxicity.

DEDICATION

For Elizabeth…

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PREFACE

I have prepared my dissertation in accordance with the guidelines set forth by the Graduate School of the University of North Carolina. This dissertation consists of a general introduction, three chapters of original data and a conclusions chapter. Each data chapter includes an abstract, introduction, materials and methods, results and discussion sections. A complete list of the literature cited throughout the dissertation has been appended to the end of the dissertation. References are listed in alphabetical order and follow the format of the journal *Toxicological Sciences.*

Papers representative of this work:

- S. Oxendine, J. Cowden, D. E. Hinton and S. Padilla (2006). Adapting the medaka embryo assay to a high-throughput approach for developmental toxicity testing. *Neurotoxicology*, in press.
- S. Oxendine, J. Cowden, D. E. Hinton and S. Padilla, 2006. Using a fish embryo assay to define vulnerable windows for developmental ethanol toxicity. (In review, *J. Aquatic Toxicology*).

Abstracts representative of this work:

- S. Oxendine, D.E. Hinton, J. Cowden and S. Padilla, (2005) Medaka fish as a model for developmental ethanol toxicity. Presented at the 22nd International Neurotoxicology meeting in Research Triangle Park, NC September, 2005.
- S. Oxendine, D.E. Hinton, J. Cowden and S. Padilla (2006) Using the medaka embryo assay to investigate critical periods for developmental ethanol toxicity. Presented at the NCSOT meeting at the US EPA, in Research Triangle Park, Durham NC February, 2006.

- Ethanol-induced teratogenicity in medaka fish (*Oryzias latipes*). S. Oxendine, R. Grindstaff, S. Padilla and D.E. Hinton. Presented at the New Directions in Graduate Research by American Indians meeting in Chapel Hill, NC October, 2005.
- Medaka early life stage tests: A rapid and reliable method for assessing temporal variations in ethanol toxicity. S. Oxendine, D.E. Hinton, J. Cowden and S. Padilla**,** Presented at the Genetics and Environmental Mutagenesis Society Meeting in Chapel Hill, NC October, 2005.
- Medaka fish as a model for developmental ethanol toxicity: Investigation of windows of heightened sensitivity. S. Oxendine, D.E. Hinton, J. Cowden and S. Padilla**,** Presented at the Society of Toxicology meeting in San Diego, CA. March, 2006.

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AC	Adenylate cyclase
ADH	Alcohol dehydrogenase
ARND	Alcohol-related neurodevelopmental disorders
BEC	Blood ethanol concentration
CAM	Cell adhesion molecule(s)
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
ERM	Embryo rearing medium
EPA	Environmental Protection Agency
EtOH	Ethanol
FAS	Fetal alcohol syndrome
GABA	γ-Amino butyric acid
GD	Gestational day
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid
IACUC	Institutional animal care and use committee
LC_{50}	Concentration that is lethal to 50% of the subjects
LD_{50}	Dose that is lethal to 50% of the subjects
MPT	Mitochondrial permeability transition
PBS	Phosphate buffered saline
PD	Postnatal day

CHAPTER 1.

INTRODUCTION

What is fetal alcohol syndrome?

Maternal ethanol abuse is the leading known, preventable cause of mental retardation in the Western world (Abel and Hannigan 1995; Abel and Sokol 1987; Chiriboga 2003), even though studies dating back to the early 1900's clearly indicate that consumption during pregnancy can adversely affect the fetus (Stockard 1910). Although Lemoine and coworkers were first to present clinical evidence of ethanol-induced fetal malformations (Lemoine *et al.* 1968), the term "Fetal Alcohol Syndrome" (FAS) was originally coined in the U.S. to describe a constellation of physical and cognitive abnormalities observed in children of alcoholic mothers (Jones and Smith 1973). Manifestations of FAS have since been described in most countries of the world. Affected individuals usually present with a spectrum of anomalies including growth deficiency, cognitive impairment, and distinctive craniofacial features (reviewed in Coles 1993; Meyer and Riley 1986; Smith 1997; Streissguth *et al.* 1986).

The association between ethanol consumption and adverse outcome during pregnancy is well established (Abel 1980; Henderson 1982; Jones *et al.* 1976; Streissguth *et al.* 1980). Diagnosis, however, has been hampered by the fact that the FAS phenotype exists along a continuum which varies considerably in terms of frequency and severity of

expression. The complete spectrum of effects (craniofacial malformations, CNS dysfunction and pre- or postnatal growth retardation) occurs in approximately 0.3% of all live births (Charness *et al.* 1989), generating an estimated lifetime expenditure of approximately $1.4 million per FAS child (Abel and Sokol 1991; Clarren *et al.* 1990; Streissguth *et al.* 1991). Although facial dysmorphology is perhaps the most striking manifestation of developmental ethanol toxicity, impaired growth is the most frequent consequence clinically and experimentally (Kennedy 1984). Intellectual impairment, which appears to be closely linked to ethanol-induced growth inhibition, has been documented in the absence of overt toxicity (Abel 1980; Clarren *et al.* 1978; Kennedy 1984).

What is the mechanism of action?

Many different mechanisms likely contribute to ethanol-induced fetal damage, although none have been established with any certainty. Recent literature reviews confirm that no single biological mechanism can account for all of the phenotypic abnormalities observed in children prenatally exposed to ethanol (Abel and Hannigan 1995; Guerri 1998; Maier *et al.* 1996; Michaelis 1990; Michaelis and Michaelis 1994; Phillips and Cragg 1982; Schenker *et al.* 1990; West *et al.* 1994). Researchers have traditionally used mammalian models and cell-culture experiments to investigate developmental ethanol toxicity. These approaches have identified a number of potential mechanisms, including mitochondrial damage and subsequent increases in oxidative stress, interference with glia, neurotrophic factors and a number of chemical signaling systems involved in neuronal communication, changes in the transport and uptake of glucose, impaired cell adhesion

and altered gene expression during development. In addition, decreased protein synthesis, impaired nutrient utilization, altered prostaglandin homeostasis, and hypoxia are other key considerations in ethanol-related growth restriction. Ethanol can exert its pleiotropic effects directly, by acting on the conceptus, and indirectly, by interfering with maternal support, making it exceedingly difficult to identify specific cause and effect relationships.

Cell Death

There are two generally accepted mechanisms of cell death, necrosis and apoptosis. These processes can be distinguished by morphological and biochemical events that occur during cell death. Cells undergoing necrosis typically swell and eventually rupture, spilling cytoplasmic debris that initiates an inflammatory response which destroys adjacent cells. Apoptosis is a form of "cell suicide" that systematically deletes individual cells, while leaving adjacent cells intact. During this highly regulated process, cell bodies shrink and nuclear DNA becomes condensed and fragmented. Eventually, cells disintegrate into “apoptotic bodies” that are engulfed and destroyed by scavenging cells (Bredensen 1996a, b). Many physiological events can activate the apoptotic pathway including ischemia, mitochondrial dysfunction, oxidative stress and disruption of calcium homeostasis. Developmental ethanol exposure is also known to cause apoptosis. This has been demonstrated both in animal models of early ethanol exposure (Cartwright *et al.* 1998) and in studies of cultured hypothalamic neurons (De *et al.* 1994).

Although diverse molecular signals can initiate cell death, activation of specific "death-promoting" effector molecules is considered to be a key regulatory event in the apoptotic pathway (Bredensen 1996a, b). Caspase enzymes cleave and destroy important

proteins in the cell, thereby initiating programmed cell death (Cohen 1997), but in some instances, cells exposed to these “cell death-inducing factors” do not die. Survival determined by the balance between pro-apoptotic and anti-apoptotic cellular constituents. Disruption of this balance in favor of proteins encoded by "cell death genes" is thought to play a role in ethanol-induced apoptotic cell death. Increased expression of anti-apoptotic members of the bcl-2 protein family, however, has been shown to prevent ethanol-induced cell death during development (Heaton *et al.* 1999).

Oxidative Stress

As previously mentioned, oxidative stress and excessive free radical production can initiate cell death via several biochemical cascades, including the release of “apoptosis-inducing factors” (Bredensen 1996a, b). Ethanol metabolism and subsequent generation of reactive oxygen species is known to reduce antioxidant levels. Accumulating evidence suggests that this phenomenon may contribute to ethanol-induced cell damage and cell death in the fetus (Guerri 1998; Henderson *et al.* 1995; Kotch *et al.* 1995). In addition, animal models have shown that treatment with antioxidants may ameliorate ethanol-induced cell damage (Heaton *et al.* 2000).

Ethanol-induced free radical production can also damage cells by interfering with mitochondrial function. Mitochondria actively sequester calcium ions, and maintain calcium homeostasis, which is vital for survival (Choi 1995). When mitochondria become dysfunctional, they undergo a process called mitochondrial permeability transition (MPT), wherein large holes open up in the mitochondrial membrane, releasing calcium

and cytochrome c into the cytoplasm. This process can cause necrosis or apoptosis (Kroemer *et al.* 1997).

Neurotransmitter Function

Ethanol is known to adversely affect the structure and function of developing neurons by interfering with various neurotransmitter systems, many of which (*e.g.*, acetylcholine, glutamate, serotonin, and gamma-aminobutyric acid) serve to orchestrate CNS organization during fetal development. For example, glutamate and NMDA receptor interactions appear to be critical for stabilizing synapses that form during brain development. Developmental ethanol exposure can reduce the number and function of NMDA receptors both during early development and during later developmental stages (Costa *et al.* 2000; Rema and Ebner 1999).

Cell Cycle Perturbations

Developmental ethanol exposure may damage the fetal brain by interfering with the generation of neuronal progenitor cells. Ethanol can decrease neurogenesis in cellular proliferative zones by slowing the cell cycle and limiting the number of newly generated cells which are able to migrate to appropriate locations and establish synaptic connections with target neurons in the mature brain (Miller and Nowakowski 1991). Ethanol is also known to interfere with growth factors that regulate cell division (Resincoff *et al.* 1993). Ethanol has been shown to induce cell death in post-mitotic cells by inhibiting growth factors that facilitate cell differentiation (Cui *et al.* 1997; Zhang *et al.* 1998a, b) and to delay the development of serotonergic networks by interfering with interactions between

developing serotonin neurons and astrocytes that support their growth (Eriksen *et al.* 2000; Kim and Druse 1996). Under normal circumstances, radial glia become astrocytes when neurons reach their final location. This transition occurs prematurely with developmental ethanol exposure (Miller and Robertson 1993), causing neurons to lose their radial glial guides, stop migrating and end up in abnormal positions that may adversely affect survival.

Excitotoxicity

Excitotoxicity is another critical determinant of ethanol-related CNS damage (Michaelis 1990; Michaelis and Michaelis 1994). Although calcium influx generated by NMDA receptor activation is an important signal for normal neuronal development, excessive activation of NMDA glutamate receptors can lead to dangerously high calcium accumulation inside the neuron (Choi 1995) and cause cell death by apoptosis or necrosis (Choi 1995; Pang and Geddes 1997; Zamzami *et al.* 1997).

Glucose Metabolism

Many harmful effects of prenatal ethanol exposure are thought to be related, either directly or indirectly, to ethanol-induced disruption of glucose utilization. Glucose is an indispensable energy source which is used in the production of a variety of cellular constituents that are necessary for survival, such as lipids, nucleic acids, neurotransmitters and steroid hormones. Short-term ethanol exposure has been shown to significantly reduce cellular glucose uptake and glucose transporter expression in cultured rat neurons (Hu *et al.* 1995). This effect has also been observed *in vivo*, where prolonged

developmental ethanol exposure was shown to reduce both glucose uptake and GLUT1 gene expression in rats (Singh *et al.* 1992). Since glucose plays a crucial role in most physiological systems, ethanol-induced dysregulation of glucose transport is a prime candidate for potential mechanisms of ethanol-induced growth inhibition and CNS damage.

Cell Adhesion

Ethanol has been shown to interfere with normal brain development by reducing cell adhesion interactions. Neurons must establish cell-to-cell contact during growth and development in order to migrate to their final destination and develop the appropriate connections which are necessary for survival. Numerous cell adhesion molecules (CAMs) assist in various aspects of this process. In humans, defects in L1 CAMs can lead to abnormal brain development, characterized by mental retardation, absence of the corpus callosum, and cerebellar malformations. These brain anomalies are similar to those found in patients with FAS, suggesting that ethanol exposure during gestation may similarly affect L1-mediated cell adhesion and thereby contribute to various aspects of the FAS phenotype. This hypothesis is supported by the fact that low doses of ethanol (*i.e.*, less than 0.05%) inhibit L1-mediated cell adhesion in cultured brain cells (Ramanathan *et al.* 1996).

Ethanol exposure produces a range of structural and functional abnormalities. Observed manifestations of FAS depend upon the dose, duration, and pattern of ethanol exposure during development. In addition, susceptibility to the deleterious effects of ethanol exposure may vary according to the specific cell type and developmental stage of

the organism at the time of exposure. Currently, there is no “global” mode of action for ethanol-induced developmental toxicity and it is highly unlikely that a single mechanism can account for all of the adverse effects that have been documented in the literature. In order to gain a better understanding of this problem, researchers must first determine how the initiation and progression of ethanol toxicity changes in relation to blood ethanol concentration (BEC) and developmental timing.

What is the relationship between timing and toxicity?

Although "critical periods" of heightened sensitivity are best documented for the dysmorphic effects of ethanol toxicity, data relating to stage-specific neurochemical changes and associated functional deficits are lacking with regard to developmental ethanol exposure. For this reason, an assessment of neurotoxic potential as it relates to the timing of ethanol exposure is warranted. Very few studies have compared ethanol toxicity at distinct periods throughout gestation, though some have looked at individual periods.

Pre-differentiation phase

Human studies of ethanol exposures that occur prior to implantation are limited due to ethical considerations. Pregnancy often remains largely undetected at this point, but spontaneous abortions have been reported following ethanol exposure around the time of conception (West *et al.* 1994). Only a handful of animal studies have examined the toxic effects of ethanol exposure during this phase of development and the results obtained from these investigations are variable. For example, in a mouse study designed to investigate the effects of developmental ethanol exposure during pre-differentiation,

mice appeared to develop normally following ethanol administration on GD 6 (Lochry *et al.* 1982). This is contrasted by findings obtained in a similar study conducted a few years later, which demonstrated that ethanol exposures on GD1-6 increased prenatal mortality (resorptions) and produced significant decreases in fetal birth weight. The latter study also reported craniofacial and limb abnormalities in over 80% of affected offspring, suggesting that early ethanol exposure may be teratogenic (or lethal) to the embryo (Padmanabhan and Hameed 1988).

Embryonic Period

The embryonic period is extremely sensitive and ethanol-induced teratogenicity has been studied extensively at this stage of development. In humans, ethanol exposure during embryonic development may produce craniofacial malformations (*e.g.*, cleft palate, microcephaly) as well as cardiac, muscular and skeletal abnormalities (Clarren and Smith 1978). A number of rodent strains have been used to investigate the effects of ethanol exposure during this developmental period, but results vary considerably. For instance, craniofacial abnormalities (Sulik *et al.* 1981; Webster *et al.* 1980), ocular defects (Cook *et al.* 1987; Webster *et al.* 1983), cardiac dysmorphology (Daft *et al.* 1986) and skeletal anomalies (Blakley and Scott 1984; Ciociola and Gautieri 1988) have all been reported following ethanol exposure during early embryonic development (GD 7/8). Urogenital (hydronephrosis) (Boggan *et al.* 1989) and forelimb malformations (Gilliam and Irtenkauf 1990) have also been noted with ethanol exposures that occur during embryogenesis (GD 9/10).

Fetal Period

Although there are some inconsistencies in the epidemiological data, human studies suggest that ethanol exposures during this phase of development generally do not produce gross structural malformations, however, they may inhibit growth and produce subtle damage within the developing CNS (*e.g.*, cognitive and neurobehavioral deficits) by interfering with synaptogenesis, myelin formation, or other critical aspects of neuronal development (West 1987). Ethanol-toxicity has also been demonstrated using whole embryo culture models. Effects observed in these studies include ethanol-related decreases in body length, DNA/protein content and brain development (Brown *et al.* 1979; Priscott 1982). Animal models have been extremely useful for investigating the effects of ethanol exposure during this particular stage of development. Investigators have demonstrated a strong correlation between ethanol exposure late in gestation and fetal growth deficiency using rat (Gavin *et al.* 1994); mouse (Blakley and Scott 1984); chick (Boyd *et al.* 1984; Pennington 1988) and non-human primate (Sheller *et al.* 1988) models. In one rodent study, ethanol exposure during the latter part of gestation (GD 14-21) or throughout gestation (GD 1-22) produced weight deficits in offspring, whereas exposure to equivalent BECs during an earlier developmental period (GD 1-13) did not (Abel and Dintcheff 1978). This observation supports the findings of (Middaugh and Boggan 1991), who used a mouse model to demonstrate that ethanol exposure late in development (GD 12-17) produced greater ethanol-related growth restriction than exposures that occurred during earlier stages of development (GD 5-10).

Which stage of development is most sensitive?

Human Studies

Although the FAS phenotype is well characterized clinically, there is still no clear consensus regarding which periods of development are most sensitive to ethanol toxicity because the available information regarding temporal vulnerability is fairly limited. The clinical literature only *suggests* that there are critical periods during development wherein the human fetus experiences heightened sensitivity to ethanol. Unfortunately there are still no definitive answers regarding temporal aspects of ethanol toxicity due to the fact that few investigators have thoroughly examined this question. A number of human studies do imply, however, that intrauterine growth retardation is associated with ethanol exposure during the latter part of gestation. Women who stopped drinking before the end of the second trimester reportedly had babies that were comparable (in terms of birth weight and head circumference) to infants of women who never consumed ethanol during pregnancy (Rosett *et al.* 1983; Smith *et al.* 1986). These studies are strengthened by subsequent reports of decreased growth inhibition in offspring of mothers who discontinued drinking before the third trimester relative to offspring of mothers who drank throughout pregnancy (Coles 1994; Jacobson *et al.* 1994). These findings are inconsistent however, with an earlier report indicating that a significant relationship exists between first trimester ethanol exposure and low birth weight (Day *et al.* 1989). This contention is consistent with results obtained in another study which demonstrated that ethanol exposure during early gestation was more damaging to cognitive function than later and considerably greater ethanol exposures (Clarren and Smith 1978). The lack

of concordance with regard to periods of heightened sensitivity for developmental ethanol exposure most likely reflects deficiencies in experimental design, as most failed to conduct a complete time course or dose response that would facilitate comparisons with exposures that encompass early, middle or late development while using a common endpoint to define toxicity.

Behavioral manifestations of developmental ethanol exposure have also been reported in humans. For example, Aronson and colleagues compared children of alcoholic mothers who consumed ethanol throughout gestation with those born to women who abstained after the twelfth week of gestation. Severe behavioral and intellectual abnormalities were noted in children exposed throughout pregnancy, whereas children exposed only through the twelfth week of gestation appeared to develop normally (Aronson *et al.* 1997). The authors suggest that this finding is indicative of increased susceptibility to developmental ethanol toxicity during the latter part of gestation. Unfortunately, this study is compromised by the fact that women who drink late in pregnancy generally tend to drink throughout pregnancy, making it virtually impossible to discern the effects of early versus late gestational exposure. In addition, women who abstain from ethanol early in pregnancy are more likely to possess other positive attributes such as better prenatal care or higher socioeconomic status which may improve outcome (Day *et al.* 1991). Hence, these types of studies, which are often used to demonstrate that drinking later in pregnancy is most detrimental to the fetus, are limited by confounding factors associated with maternal influence. Therefore, human evidence in support of critical periods for enhanced sensitivity to developmental ethanol toxicity is insufficient. As such, questions regarding how timing affects developmental ethanol

toxicity need to be revisited using animal models that incorporate the appropriate controls and facilitate delivery of precise dosages during clearly defined stages of development.

Non-Human Primate Studies

Although many early reports suggest that the CNS is most sensitive to developmental ethanol exposures that occur during the human third trimester equivalent, recent studies have challenged this notion. For instance, Clarren and co-workers have suggested that ethanol exposures which occur during early gestation are more damaging to cognitive function than later ethanol exposures. These investigators used a non-human primate model to show that consumption of as little as six drinks per week (~1.8 g/kg) early in gestation was sufficient to produce deficits as severe as those elicited from drinking throughout pregnancy (Clarren *et al.* 1992). This finding is consistent with human studies which have shown a strong correlation between ethanol consumption at conception and neurobehavioral impairments (Schneider *et al.* 2001; Streissguth *et al.* 1989). Inconsistencies in the clinical and experimental literature most likely reflect the complexity of physiological interactions between timing and toxicity that impact numerous cell signaling pathways, producing a diverse spectrum of effects. Nevertheless, these reports cast serious doubt on the supposition that the human third trimester equivalent is the most sensitive period for developmental ethanol exposure.

Rodent Studies

In rodents, the early postnatal period (*e.g.*, human third trimester equivalent) is generally considered to be the most vulnerable period for ethanol-induced CNS damage

(West *et al.* 1986). For example, in one study ethanol exposure during gestation produced no effect on hippocampal mossy fibers, whereas exposures that occurred during the early postnatal period dramatically decreased both hippocampal mossy fiber organization and cerebellar neuronal density (Hamre and West 1993; West and Hamre 1985). Further support for this notion of differential sensitivity was provided by decreases in Purkinje cell numbers following early postnatal ethanol exposure (PD 3/4) (Bauer-Moffett and Altman 1977) that were not observed when ethanol exposure occurred later in development (PD 10) (Pauli *et al.* 1995). Another research group used behavior, brain weight and the brain:body weight ratio to compare ethanol sensitivity across early, middle and late development (Tran *et al.* 2000). In this study, ethanol exposure produced more adverse effects in rats treated late in development. These investigators were careful to equate peak blood ethanol concentrations across all exposure periods, suggesting that the observed temporal variations in ethanol toxicity reflect differences in developmental timing rather than ethanol concentration. Therefore the issue of "critical windows" for developmental ethanol toxicity is as yet, unresolved.

Why Medaka?

Although rodents have been invaluable for conducting experiments that cannot be performed in humans, they share a major experimental weakness with humans. Their capacity for placental reproduction introduces uncertainty and limits the number of embryos that can be produced by each female. In addition, rodent embryos are microscopic early in gestation and require sacrifice of the mother in order to gain access to them. Another problem with placental reproduction is that treatment-related mortality

may go undetected because embryos that die during development are rapidly resorbed, making them unavailable for assessment. These issues are compounded by the fact that costs associated with rigorous toxicity testing *in vivo* may be prohibitive when using rodent models.

Approximately 15% of the animals used for toxicological studies are devoted to the identification of chemical hazards to human development (Bremer *et al.* 2005). Use of fish as alternative models has grown considerably due to mounting socioeconomic pressures against animal testing (Ishikawa 2000a). Due to the fact that steroid hormone structure and function are highly conserved between fish and mammals, a variety of fish species are now being used as models of endocrine disruption both in field and laboratory settings (Bortone and Davis 1994; Folmar *et al.* 1996; Gimeno *et al.* 1998; Ota *et al.* 2000). Fish models have been used to assess a range of mammalian responses to toxicant exposures, including carcinogenesis (Hinton *et al.* 1985), endocrine disruption (Kashiwada *et al.* 2002), mutagenesis (Mullins *et al.* 1994), oxidative stress (Kelly *et al.* 1998) and immunotoxicity (Gogal *et al.* 1999). Although best known for their utility in large scale mutagenesis screens, zebrafish *(Danio rerio*) have been extensively studied since the early 1930s and have remained a focal point in developmental biology for several decades (Hisaoka 1958). A detailed review of their biology and laboratory use is presented by (Laale 1977). These small tropical fish, indigenous to East India and Burma, have been widely used as experimental models for studies of vertebrate development and genetics (Parng *et al.* 2002). This is due at least in part, to their economic husbandry requirements, high reproductive capacity and rapid generation cycle. In addition, axogenesis and morphogenesis of early brain structures are

well characterized in zebrafish (Chitnis and Kuwada 1990; Kimmel *et al.* 1995). Detailed methods for zebrafish culture in the laboratory are available (Westerfield 2000).

The Japanese medaka (*Oryzias latipes*) has a vast research history compared to zebrafish and a small genome size (~800-1000 Mb) which decreases redundancy (Naruse *et al.* 2004). This fresh water killifish which is indigenous to Japan, Taiwan and Asia, has been studied for over a century and has over 1000 research references dating back to the early 1900s, making it one of the most extensively characterized fish models in history. Many critical events in development are highly conserved across species. As such, medaka embryology and brain structure are remarkably similar to human. (Ishii 1967) conducted the first morphological analysis of medaka brain development and demonstrated that the major structures of the forebrain are well conserved across phyla. Since then, many neuroanatomical and developmental studies of the medaka CNS have been conducted (Ishikawa and Hyodo-Taguchi 1994; Koster *et al.* 1997; Kuwada 1986). A complete gross and micro-anatomical description of the medaka brain has been published (Ishikawa *et al.* 1999; Iwamatsu 2004).

Since zebrafish are a well established model of developmental biology (Fishman 2001), a general comparison between medaka and zebrafish may serve to illustrate the utility of medaka early life stage testing. In zebrafish, more than 500 mutant phenotypes have been identified which may prove useful for studying various aspects of early development. Medaka are hardy fish, as evidenced by the fact that they can tolerate low oxygen tensions and a wide range of pH, temperature and salinity requirements which are known to adversely affect embryonic development and survival in zebrafish (Yamamoto 1975). Some of the advantages shared by these two models include: low space

requirements, a small stature (3-5 cm), which facilitates thorough histological sampling at a fraction of the cost generated using rodent bioassays and a transparent outer chorion which allows for detailed, non-invasive observation of toxicant effects throughout development. In addition, medaka fish have enormous reproductive potential, making it possible to generate large sample populations quickly, thereby increasing statistical power. Individual females reach sexual maturity within 6-8 weeks (compared to 10-12 weeks in zebrafish) and lay eggs year round (~25-30 eggs/day/female), generating up to ten thousand rapidly developing embryos per year (Hirshfield 1980). Medaka and zebrafish develop *ex utero* and exhibit optical clarity throughout embryogenesis, allowing egg fertilization and subsequent developmental processes to be assessed easily with a dissecting microscope. When embryos are incubated at 26°C, the length of gestation is approximately 9 days in medaka and 3 days in zebrafish. The more protracted duration of medaka development facilitates investigations that would otherwise be difficult to execute during the relatively brief time period required for completion of embryonic development in zebrafish.

As vertebrates, medaka and zebrafish offer distinct advantages over other models that have typically been used for large scale screening, (*e.g.*, *Drosophila melanogaster* and *Caenorhabditis elegans)*, by providing a more comparative model for mechanistic studies. Since many of the zebrafish mutant phenotypes identified in genetic screens thus far are reminiscent of human disease states, this model may provide useful insights for corresponding pathophysiology. A recent review includes several examples of clinically relevant zebrafish mutants and also describes molecular and genetic techniques that are being used to build the zebrafish genomic infrastructure (Dooley and Zon 2000). Medaka

and zebrafish are particularly well-suited for mechanistic studies of development. Both genomes have been completely sequenced, making it possible to integrate toxicant effects at cellular, molecular and functional levels when investigating the level of concordance between fish and mammalian responses to toxicant exposure.

While small aquarium fish models represent a convenient alternative for developmental toxicity testing, there are a number of important issues to consider, as they may influence physiological responses. For instance, healthy test animals are essential for obtaining repeatable results and limiting confounding factors that could contribute to variability or produce spurious data. For example, environmental stressors, like hypoxia or poor water quality can adversely affect viability and thereby impact toxicity. High salinity conditions have been shown to enhance the negative effects of certain compounds (Dyer *et al.* 1989), whereas increased water hardness may have a protective effect due to decreased diffusion across gill epithelia (Noga 1996). Changes in presystemic metabolism (Barron *et al.* 1988) or specific biotransformation enzymes may also limit bioavailability (and toxicity) of certain test compounds (Karr *et al.* 1985). Our ability to extrapolate between fish and other species is further complicated by interspecies variability in chemical transport, limitations in the amount of blood (or tissue) available for assessments and uncertainty regarding variations in organ and/or metabolic function. In addition, fish lack structural elements, such as hair, gonads and mammary glands etc., normally found in mammals, which may restrict comparative approaches.

Despite various technical limitations, use of fish as sentinels of toxicity has grown in popularity (Ishikawa 2000a; Yamamoto 1975). The relative ease with which large numbers of animals can be cultured in a limited space has facilitated a wide range of

studies including those addressing behaviorial (Bryant and Grant 1995; Howard *et al.* 1998; Magnuson 1962), pigment (Koga *et al.* 1999; Oshima *et al.* 1998; Sugimoto and Oshima 1995), vision (Hisatomi *et al.* 1998; Nguyen *et al.* 1999; Nishiwaki *et al.* 1997; Seimiya *et al.* 1997), and genetic research (Koga *et al.* 1996; Krause *et al.* 1997; Nonaka *et al.* 1998; Takeuchi 1966; Tanaka *et al.* 1995; Yasuoka *et al.* 1995).

The basic metabolic machinery in fish, (*e.g.*, Phase I and Phase II metabolism) is similar to that of mammals, although current evidence suggests that only a few of the medaka cytochrome P450 enzyme systems (*e.g.*, CYP1A, CYP2E1) are induced by environmental toxicants (Lipscomb *et al.* 1998; Williams *et al.* 1998). Medaka are sensitive to a variety of known carcinogens and require only a short time interval for the induction of tumorigenesis (Hawkins *et al.* 1998). In addition, the mechanisms underlying carcinogenesis and mutagenesis in medaka are remarkably similar to that of human (McElroy *et al.* 2006) making the medaka model an ideal complement for rodent studies of known or suspected carcinogens.

Fish and mammalian systems share chemical and species-specific responses to xenobiotic exposure and therefore manifest comparable toxicities (Bunton 1996). As such, lower vertebrate models have the potential to rapidly advance our understanding of the basic physiological processes underlying adverse health effects. The identification of specific human disease phenotypes in fish has increased confidence in their feasibility as an alternative model and secured their standing as important tools for developmental toxicity testing. Zebrafish have historically been used for genetic and molecular studies of developmental biology, whereas medaka have primarily been used for toxicity testing of various life stages (*e.g.*, embryos, hatchling fry, and juvenile or adult fish) (Hinton *et al.*

2005). Medaka are, however, now increasingly being used as a model for studies of vertebrate genetics and mutagenicity (Ishikawa 2000b; Loosli *et al.* 2000; Nguyen *et al.* 1999; Shima and Shimada 1994). The high fecundity and short generation time which is characteristic of medaka make these small fish particularly well-suited for large-scale screening applications. In addition, the external development of relatively large eggs with large pronuclei has made medaka a good model for transgenic (Chen and Powers 1990; Ozato *et al.* 1986; Takagi *et al.* 1994; Toyohara *et al.* 1996) and fertilization studies (Yamamoto 1961). Accurate determination of specific sites for embryonic and larval injury secondary to xenobiotic exposure is now possible using early life stages of medaka development (Hamm *et al.* 1998). In addition, medaka have routinely been cloned using a protocol involving deactivation of sperm that serve to activate, but not fertilize, eggs from a single female (Naruse *et al.* 1985). This procedure facilitates the development of identical animals, which is ideal for the control of genetic influences.

Japanese researchers have developed a number of inbred medaka strains which can be used as tools for genetic research (Hyodo-Taguchi and Egami 1985). Inbred strains have also been used in radiation biology and carcinogenesis studies (Hyodo-Taguchi and Egami 1989). Because medaka inbred strains provide a more homogenous genetic background, they offer advantages over outbred strains for the control of experimental variability in a broad range of research applications. While use of out-bred strains may well reflect the range of possible responses in a heterogeneous population, use of inbred strains facilitates standardization and homogeneity of outcome, which is invaluable for mechanistic studies. Both medaka and zebrafish models are optimal for *in vivo* assessments of developmental toxicity and their shortened life span make them an ideal alternative for investigating acute and chronic, sub-

lethal effects (Ensenbach and Nagel 1995). The inherent sensitivity to chemical exposures, the ease with which large sample numbers can be quickly obtained for study and the ability to control experimental and extraneous factors make fish models an excellent choice for investigating mechanisms of developmental toxicity.

Goals of this dissertation

The overall goal of this research was to determine how dose and timing impact toxicity in an isolated, self-sustaining organism which develops *ex utero*. An investigation of the effects of ethanol exposure on early life stages of the Japanese medaka (*Oryzias latipes*) eliminates confounding factors associated with maternal influence, allows for accurate dosing and precise timing of exposures, and provides an intact nervous system for various structural assessments. Experiments described in this dissertation investigated the effects of developmental ethanol exposure in medaka embryos using the following hypothesis: The timing of developmental ethanol exposure will influence manifestations of ethanol toxicity in medaka embryos, as outlined in the following Aims.

Aim 1 Investigated manifestations of developmental ethanol toxicity in medaka embryos.

Aim II Identified windows of enhanced sensitivity to developmental ethanol toxicity in medaka embryos.

Aim III Determined the correlation between critical periods for heightened sensitivity to developmental ethanol toxicity and apoptotic changes within the medaka brain.

CHAPTER 2.

ADAPTING THE MEDAKA EMBRYO ASSAY TO A HIGH-THROUGHPUT APPROACH FOR DEVELOPMENTAL TOXICITY TESTING

In press, *Neurotoxicology*, 2006

Preface

As I began the experiments designed to study developmental ethanol toxicity, a series of technical issues had to be overcome. This chapter (now in press) describes a 96-well microtiter plate adaptation of the medaka early life stage assay which I developed to facilitate a reliable, "semi-high throughput" approach for developmental toxicity testing.

Abstract

Chemical exposure during embryonic development may cause persistent effects, yet developmental toxicity data exist for very few chemicals. Current testing procedures are time consuming and costly, underlining the need for rapid and low cost screening strategies. Although *in vitro* methods are useful for screening, these methods do not replicate all the intricacies which are characteristic of embryonic development and should ideally be complemented by an *in vivo* screening strategy. In this study, a modification of the medaka embryo assay was devised in order to meet the requirements of high-throughput, developmental toxicity testing *in vivo*. The Japanese medaka (*Oryzias latipes*) offers several advantages over traditional mammalian model systems, including economic husbandry, high fecundity, and rapid *ex utero* development. In most studies where fish eggs are exposed to a chemical, toxicant exposure takes place in a common vessel, with many embryos being exposed to the same solution. This type of design is not amenable to high-throughput methodologies, does not allow the investigator to follow the same embryo throughout gestation and may confound statistical analysis of the results. A 96-well microtiter plate method was used to facilitate exposure of individual medaka embryos within single wells and this approach was compared with the common vessel method using the industrial solvent dimethyl sulfoxide (DMSO) as a test compound. At lower DMSO concentrations (0 or 1%), the 96-well microtiter plate assay replicated results that were obtained using the common vessel method. There was, however, increased lethality and decreased hatching rate in the bottle-reared embryos treated with higher DMSO concentrations (5% or 10%). Since embryos reared in the 96-well microtiter plates never showed an increase in adverse effects (compared to the bottle-

reared embryos) at any DMSO concentration, this suggests that the 96-well microtiter plate assay may provide a rapid and efficient alternative for developmental toxicity screens that utilize fish embryos.

Introduction

Embryogenesis is highly sensitive to toxicant exposure, yet many chemicals currently registered for commercial use lack comprehensive safety data. Considering the number of compounds that need testing, novel methods are required to expedite reliable hazard identification and to enhance the predictive power of developmental toxicity studies (Bantle *et al.* 1988; Benoit *et al.* 1982; Birge *et al.* 1985; Cameron *et al.* 1985; Dresser *et al.* 1992; Hawkins *et al.* 2003; Law 2003; Sabourin and Faulk; Weis and Weis 1987). Various *in vitro* assays have been useful for screening putative toxicants, but these tests cannot incorporate all of the complexities of mammalian development. It would be optimal to augment *in vitro* testing with *in vivo* assays to provide a more complete assessment of neurotoxic potential following developmental exposure to a given chemical.

Japanese medaka fish (*Oryzias latipes*) offer several advantages for developmental toxicity testing, including economic husbandry requirements, high fecundity, and rapid *ex utero* development. Further, the transparent outer chorion facilitates non-invasive observations of the embryo throughout development (Braunbeck *et al.* 2004; Hawkins *et al.* 2003; Wittbrodt *et al.* 2002). In addition, medaka embryos are known to be sensitive to toxicant exposure during development (*e.g.*, Dial 1978; González-Doncel *et al.* 2003; Smithberg; Solomon and Faustman 1987; Villalobos *et al.* 2000). These attributes make medaka an attractive candidate for high-throughput screening.

In this study, I explored the utility of medaka embryos as a candidate for developmental toxicity screens. In order to facilitate the use of robotics in these types of screens, a multi-well plate format was adopted for toxicity testing. This method is

preferable to the "common vessel" method which is typically used for embryo toxicity assays. Specialized 96-well microtiter plates (described below), were used to compare the conventional method of exposing several embryos in a single rearing environment (*e.g.*, borosilicate bottles) to the modified, 96-well microtiter plate assay. To accomplish this task, medaka embryos were exposed to various nominal concentrations of dimethyl sulfoxide (DMSO) (Jacob and Herschler 1986), a commercial solvent commonly used as a vehicle in toxicity studies. All embryos were assessed daily for lethality, hatching rate and day of hatch. Comparison of the 96-well Multiscreen[J] microtiter plate method with the "common vessel" method showed that, in general, both treatment scenarios produced similar results, suggesting that the 96-well microtiter plate strategy is a suitable alternative to the borosilicate bottle method commonly used in fish embryo toxicity testing. Moreover, the 96-well microtiter plate method adds individual, serial, evaluative and statistical strengths that cannot be obtained with conventional approaches.

Materials and Methods

Chemicals

Embryo rearing medium (ERM, 17.1mM NaCl, 272µM $CaCl_2 \bullet 2H_2O$, 402µM KCl, 661µM $MgSO_4 \bullet 7H_2O$; pH 6.3) was used as the medium for all exposures. Dimethyl sulfoxide (DMSO, 99.9% purity) was purchased from Mallinckrodt AR© ACS (Phillipsburg, N.J.).

Test animal

The Japanese medaka is a small (3–4 cm) egg laying freshwater fish native to Japan, Korea, and eastern China. Embryos used in this study were collected from an orange-red line maintained under standard re-circulating aquaculture conditions. All procedures were approved by the Duke Institutional Animal Care and Use Committee (IACUC) and the U.S. EPA National Health and Environmental Effects Laboratory IACUC.

Embryo collection

Egg collection and broodstock maintenance followed the experimental procedures described by (Marty *et al.* 1991). Medaka broodstock of the orange-red variety were fed a commercial ration (Otohime Beta, Nisshin Feed Co. Ltd., Tokyo) *ad libitum* and supplemented with brine shrimp nauplii for five days each week under a 16:8 hr light/dark cycle at 25 ∀ 1° C**.** Brine shrimp nauplii were reared at 25 ∀ 1° C using a secondary container of Instant Ocean (Marineland, Moorpark, CA) under continuous aeration. For supplemental feedings, hatched brine shrimp nauplii were taken from this secondary container and 1 ml aliquots were fed to the medaka broodstock. Fertilized eggs from at least 50 females were siphoned from aquaria within 6 hours of fertilization and separated by gently rolling egg clusters by hand to disrupt attaching filaments. Eggs were pooled and washed briefly in 20 ppm Instant Ocean (Marineland, Moorpark, CA) to inhibit fungal growth. Fertilized eggs were then placed in ERM and staged according to Iwamatsu (1994). Embryos of the early high blastula stage (stage 10) were selected for study. Embryonic development was examined non-invasively through the transparent

chorion (*i.e.*, egg shell membrane) using a Nikon 1500 stereo dissecting microscope (Nikon, Melville, NY).

Experimental Design

The experimental design for these studies is shown in Figure 2-1. Two treatment methods (*i.e.*, 20 ml borosilicate bottles or 96-well microtiter plates) were used to evaluate DMSO toxicity in the medaka embryo assay. To prevent sample bias, embryos from collected clutches were pooled, then individual stage 10 embryos were randomly assigned to either 20 ml borosilicate bottles or individual wells of microtiter plates. Therefore, for each experiment, all embryos in the bottles or the microtiter plates were taken from the same mating pairs. Both exposure methods used a total of 10 embryos per dose. A stock solution of 10% DMSO in ERM (v/v) was serially diluted using ERM to prepare 1% and 5% stock solutions for developmental exposures. Sufficient quantities of each DMSO stock solution were prepared to ensure that all exposures used the same original stock solutions throughout treatment. Control embryos (exposed to ERM only) were included as a reference of normal embryonic development (Solomon and Faustman 1987). After the 7-day treatment period, embryos were maintained in ERM until hatching. Treated and control embryos were assessed daily for mortality and hatching delays. This study was done in three cohorts, each started on a different day, with a total n=20 embryos per DMSO group in each cohort. The data presented here are from all three cohorts combined.

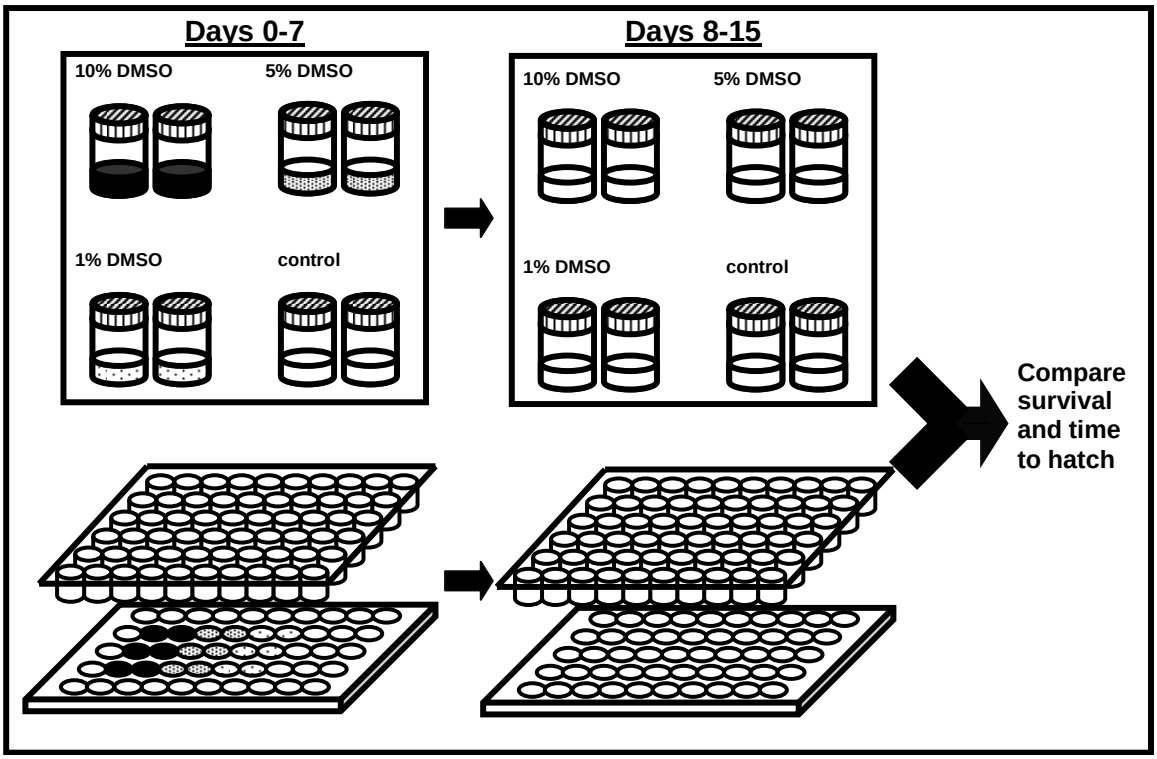


Figure 2-1 – Experimental Design Overview. *Beginning on Day 0, four DMSO solutions (10%, 5%, 1%, and 0%) - were administered to medaka embryos. For the 20ml borosilicate vial exposures, 2 groups of 5 embryos were exposed to each DMSO concentration. In the 96-well microtiter plates, 10 embryos were exposed for each DMSO concentration, all on the same plate. Solutions were changed daily from Day 0 until Day 7. Beginning on Day 8 and continuing until Day 15, embryos were stored in ERM and monitored for hatching rate and lethality.*

***Borosilicate bottles*:** Viable embryos from pooled clutches were randomly distributed into 20 ml screw-capped, borosilicate bottles (catalog 6-74516-20-CS, Krackeler Scientific, Albany, NY). Each bottle contained 5 embryos in 2 ml of solution and 18 ml of headspace, an approach adapted from (Villalobos *et al.* 1996). DMSO exposures (0, 1%, 5% or 10%) were conducted at 25 ∀ 1° C under a 16:8 hr light:dark cycle, and all solutions were renewed daily by aspirating the old solution with a glass Pasteur pipette and adding 2 ml of the original stock solution.

96-well microtiter plates: Multiscreen™ (40 micron nylon mesh) microtiter plates (catalog #MANMN4050, Millipore Corp., Bedford, MA) were used for the medaka

embryo assay. As shown in Figure 2-2, these plates consist of three separate components: a buffer tray, a sample tray, and a lid. Individual wells within the buffer tray effectively compartmentalize each embryo within its own microenvironment. The sample tray has nylon mesh at the bottom of each well. This feature permits rapid solution replacement by transferring the sample tray to a new buffer tray. Each sample tray may contain up to 60 embryos, with one embryo per well.

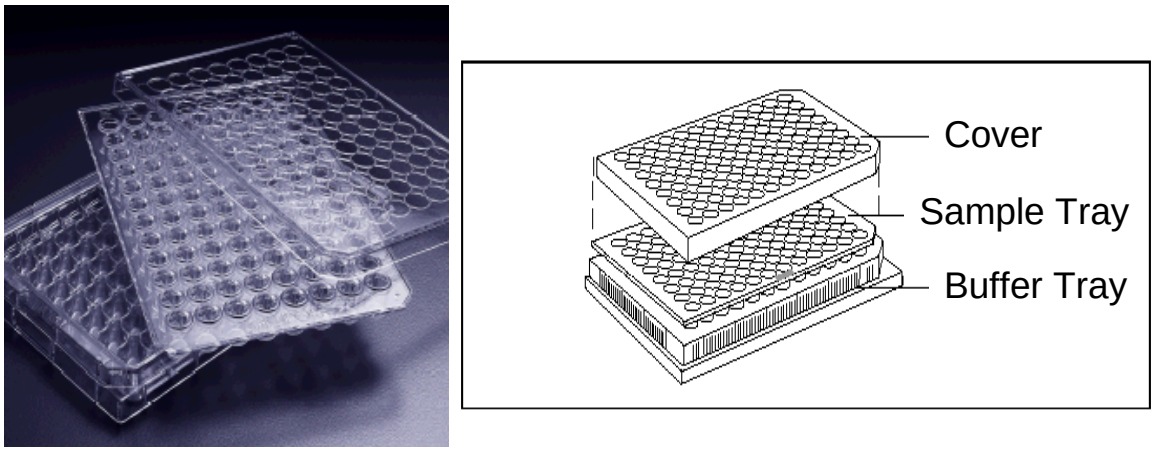


Figure 2-2 – Diagram of 96-well Microtiter Plate. *A diagrammatic representation showing the lid, sample tray, and buffer tray of the 96-well microtiter plate used for DMSO exposures. Embryos were maintained in the same sample tray throughout the experiment and solutions were changed by placing the sample tray into freshly prepared buffer trays.*

In these studies, embryos were placed in a sample tray and assigned to either experimental (DMSO solutions) or control (ERM) groups. All embryos were pooled from the same mating pairs on the same day and randomly distributed into the sample tray. In a second buffer tray, 300Φl of ERM or DMSO test solutions were pipetted into each well. In addition, 300Φl of ERM was loaded into the remaining wells located around the outer perimeter of the buffer plate to mimimize evaporation effects. Each 96-well microtiter plate was then sealed with an impermeable adhesive (Microseal®, MJ Research Inc. Waltham, MA), covered with the supplied lid, and wrapped in Parafilm® to minimize

evaporation. Medaka embryos were incubated at 25 ∀ 1° C under a 16:8 hr light:dark cycle until hatching and all solutions were renewed daily. For daily solution changes, the sample tray housing embryos was removed, briefly blotted with a paper towel and then lowered into a second buffer tray containing new aliquots of DMSO or ERM stock solutions.

Endpoints

Viability was determined through visual inspection of the medaka embryos using a Nikon 1500 stereo dissecting microscope (Nikon, Melville, NY). Death was defined as either the lack of a visible heartbeat or an egg which had become opaque. Hatching rate was defined as complete hatching: embryos with heartbeats that were partially hatched and unable to exit the chorion fully were scored as 'not hatched.' To determine the day of hatch, embryos were checked once daily, and if fully hatched, that date was recorded as the day of hatch (the day of fertilization was considered day 0).

Statistical Analyses

The two, non-parametric dependent variables (lethality and hatching rate) were analyzed using an unpaired, two group (borosilicate bottle *vs.* 96-well microtiter plate) Mann-Whitney test (StatView`J`). The parametric data (*i.e.*, length of time the embryo remained in the egg), were analyzed using a global ANOVA (Statview`J`) using day of hatch as the dependent variable, and dose and Plate or Bottle as the independent variables. Any subsequent interactions at the $p \leq 0.05$ level were followed by step-down ANOVAs. When necessary, a

Fisher's PLSD *post hoc* test was conducted to determine differences between DMSO dosage groups.

Results

The bottle-reared and 96-well microtiter plate-reared embryos showed no observable differences from one another with regard to lethality, hatching rate, or day of hatch when treated with no DMSO or 1% DMSO (Table 2-1; Fig. 2-3). The borosilicate bottles and the 96-well microtiter plates both demonstrated over 93% survivability and hatching rate in the controls and the 1% DMSO treatment groups (Table 2-1). The length of gestation (*i.e.*, hatching day) was also similar between the borosilicate bottle or 96-well microtiter plate-reared embryos: bottle = 8.7 ± 0.4 days and 96-well microtiter plate = 9.3 ± 0.2 days for the controls, and bottle = 9.3 ± 0.3 days and 96-well microtiter plate = 9.1 ± 0.3 for the 1% DMSO treatment groups (all data are presented as mean ± standard error of the mean) (Fig. 2-3). Both assays produced comparable results, indicating that rearing embryos in the 96-well microtiter plates did not enhance the negative consequences of DMSO toxicity when compared to the bottle-rearing method.

Table 2-1: Comparison of survival and hatching rate in bottle- or 96-well microtiter plate-reared medaka embryos exposed to different concentrations of DMSO.

Concentration of DMSO (%)	Survival (%)		Hatching Rate (%)	
	Bottle	96-Well Microtiter Plate	Bottle	96-Well Microtiter Plate
0	96.7 (30)*	98.3 (53)	100.0 (29)	98.3 (52)
1	93.3 (29)	96.7 (29)	100.0 (27)	100.0 (26)
5	96.7 (29)	86.7 (30)	53.3 (29)	91.7[a] (26)
10	0 (30)	63.3[a] (30)	NA	33.3[a] (19)

*The hatching rate is calculated only for the animals that survived, e.g., at 10% DMSO, only 33.3% of the 63.3% of 96-well microtiter plate reared embryos hatched. Therefore only 21% (0.333 x 0.633) of the original number of embryos reared in the 96-well microtiter plates exposed to 10% DMSO hatched. These are non-parametric data presented as means. Comparisons were made using a Mann-Whitney test. *The number in parentheses is the number of embryos observed to calculate the percent survival or percent hatching. [a]Indicates significant difference between the 96-well microtiter plate- and bottle-reared embryos.*

At higher DMSO concentrations (5% or 10%), differences emerged between the two exposure methods. Lethality was not different between the borosilicate bottle- and the 96-well microtiter plate-reared embryos at 5% DMSO, but at 10% DMSO all the embryos reared in the bottles died, whereas only 37% of the embryos reared in the 96-well microtiter plates died (Table 2-1). Comparison of hatching rate with 10% DMSO is not possible because 100% lethality at that dose was observed in the bottle-reared embryos. At 5% DMSO, however, only 53% of the embryos reared in the borosilicate bottles hatched, whereas there was a much higher hatching rate in the 96-well microtiter plate reared embryos (92%, see Table 2-1). Comparing length of gestation between the two rearing methods revealed similar patterns: embryos reared in 1% DMSO did not demonstrate delayed hatching, whereas embryos reared in 5% DMSO were delayed (Fig. 2-3). Although both the bottle-reared and 96-well microtiter plate-reared embryos showed

delayed hatching at 5% DMSO, this effect was exaggerated in embryos reared in the bottles when compared to the 96-well microtiter plate-reared embryos (Fig. 2-3).

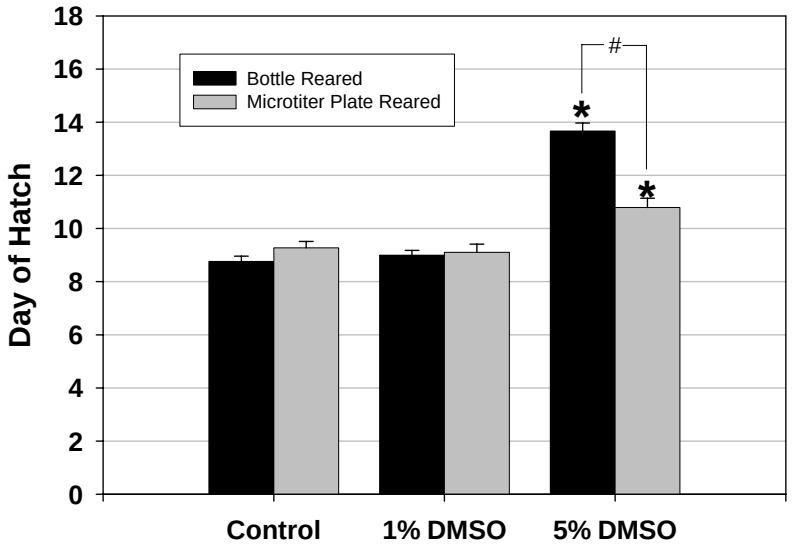


Figure 2-3 - Effects of Exposure Method on Length of Gestation*. Comparison of the length of gestation in medaka embryos reared in bottle or 96-well microtiter plates and exposed to different concentrations of DMSO. Data are presented as mean ± standard error of the means. Data were analyzed using a global ANOVA (Statview™) using day of hatch as the dependent variable, and DMSO Dose and Plate or Bottle as the independent variables. This global ANOVA indicated an interaction between DMSO Dose and Plate or Bottle ($p \leq .001$) with significant effects of DMSO Dose or Plate or Bottle alone. Step down ANOVAs indicated that (a) 5% DMSO delayed hatching in both the bottle-reared and 96-well microtiter plate-reared embryos and that (b) at 5% DMSO, the bottle-reared embryos took longer to hatch than the 96-well microtiter plate reared embryos ($p \leq .001$). * Indicates difference between that group and the control and 1% DMSO groups reared in the same manner. # Indicates that the bottle-reared embryo gestation length is different from the 96-well microtiter plate-reared embryo gestation length.*

Discussion

Early life stage toxicity tests in fish are typically carried out under group rearing conditions. The practice of exposing multiple embryos to the same solution in the same chamber diminishes statistical power. This approach also obviates serial analysis of individual embryos throughout the exposure. Furthermore, as is discussed later, this

procedure may introduce undefined inter-embryonic factors that possibly influence toxicity. Herein I describe a rapid technique for developmental toxicity testing that circumvents these issues by exposing individual embryos under isolated rearing conditions using 96-well microtiter plates. This adaptation enables large sample populations to be tested quickly, independently, and with enhanced strength of analysis. The specialized 96-well microtiter plate format allows cultured embryos to remain in their individual test wells throughout the experimental procedure. In contrast, rearing multiple embryos per bottle or Petri dish makes it impossible to follow specific embryos with any certainty during gestation. Moreover, when using pipettes to change the incubation fluid it is easy to lose embryos, or at the very least, to stress embryos when they are inadvertently taken up into the pipette tip with the exposure solution. These considerations are alleviated via use of the two-tiered, 96-well Multiscreen[J] microtiter plates.

As a test case, comparative studies using DMSO exposure of medaka embryos were conducted to assess the similarity between these two treatment paradigms. Our findings indicate that at lower DMSO concentrations, toxicity assessments conducted in borosilicate bottles were comparable to those conducted in 96-well microtiter plates, suggesting that 96-well microtiter plates represent a comparable alternative. It is interesting to note, however, that at the higher DMSO concentrations, overt toxicity (*i.e.,* death and decreased hatching rate) was accentuated in embryos that were reared in borosilicate bottles (Table 1). This observed increase in toxicity at higher DMSO concentrations may reflect undefined interactions between embryos reared in a common environment, possibly through the release of factors which may negatively impact

survival. In this scenario, if one embryo in a bottle dies, it may be more likely that other embryos in the same bottle may become predisposed to death. In fact, there is some evidence for a "group-rearing effect" on fish development. Fish embryos raised in a clutch showed a different neuroendocrine phenotype than embryos raised individually (Nechaev *et al.* 2000). Interestingly, individual fish embryos will attain a "group-reared" phenotype when incubated in water used to rear a clutch of embryos. These results suggest that a factor released into the water surrounding a clutch may influence embryonic development. An alternative explanation for the observed differences in toxicity could be an interaction between DMSO and the containment matrix of the two exposure mediums. For instance, the DMSO may be binding to the nylon mesh or polystyrene of the microtiter plate, thereby decreasing the effective concentration seen by the embryo. In that case, however, the DMSO concentration at which death was first observed would be lower in the bottle-reared embryos, which was not observed. At 5% DMSO, both the bottle-reared and 96-well microtiter plate-reared embryos showed no lethality, with death observed only at 10% DMSO. Future studies will include a determination of the actual concentration of test agent in solutions before and after incubation in 96-well microtiter plates. Another difference between the two exposure methods was available head space. In our experimental conditions, the bottles have a head space ratio of 9:1 (18 ml to 2 ml solution), whereas the 96-well microtiter plates have a ratio of only 0.33:1.00 (0.1 ml to 0.3 ml solution). Because Marty and coworkers (Marty *et al.* 1990) determined oxygen requirements in rearing solution of borosilicate vials and adjusted head space ratio accordingly, it is possible that a decrease in head space may affect oxygenation of developing tissues. Less head space in the 96-well microtiter plates,

however, did not affect the development in any of the control embryos and did not increase the toxicity at any of the DMSO concentrations used in this study. It must be noted, however, that the solutions were changed daily with aerated ERM or aerated ERM plus DMSO. Thus, the lack of head space could be detrimental to embryos reared in the 96-well microtiter plates if solutions are not renewed daily.

Other investigators have assessed the effects of DMSO on embryonic development because DMSO is often used as a cryoprotectant for eggs and embryos of many different species (Iida 1992; Johnson and Pickering 1987; Mochida *et al.* 2000; Shaw *et al.* 1991). When used as a cryoprotectant, however, the DMSO concentrations tend to be much higher than those tested here. Previous studies in frog embryos (*Xenopus*) (Dresser *et al.* 1992) or medaka embryos (Routray *et al.* 2002) exposed to DMSO during development showed a higher sensitivity to DMSO-induced lethality than was observed in the present study. For example, the LC_{50} for DMSO in frog embryos was 1.92% (Dresser *et al.* 1992), whereas 0.6 M (approximately 4.25%) DMSO was lethal to 40%-50% of the medaka embryos after a brief 20 minute exposure at either the 8-cell or "eyed" stages of embryonic development (Routray *et al.* 2002). In contrast, our data showed no lethality at 5% DMSO when exposure occurred from approximately 10 hours post fertilization through 7 days post fertilization. Routray and coworkers (2002), however, did not see a dose response relationship with regard to DMSO-induced lethality in medaka embryos; even though the DMSO concentration was increased 4-fold, lethality only increased from 40% to 50%. This result may be because, curiously, the internal concentration of DMSO also did not show a marked dose-related increase. For comparison with the present data, it would have been much more informative if lower DMSO concentrations had been used to

define the non-lethal portions of the DMSO dose response curve. In a related paper studying the effects of 0.04% DMSO on zebrafish embryos, this concentration of DMSO did not induce lethality, delayed hatching or decreased hatching rate (Hallare *et al.* 2004)

The data presented in this report clearly demonstrate that microtiter plate assays are a viable option for medaka embryo toxicity tests. This convenient method provides the necessary framework for creating an automated, high throughput screening system that will facilitate the use of robotics to investigate toxicant effects on embryonic development.

CHAPTER 3.

USE OF A FISH EMBRYO ASSAY TO DEFINE VULNERABLE WINDOW OF ETHANOL DEVELOPMENTAL TOXICITY

This Chapter and portions of Chapter 4 are currently under peer review in *Aquatic Toxicology*, 2006.

Preface

This chapter addresses the first two Aims of my dissertation and investigates temporal changes in ethanol sensitivity during specific periods of medaka development. In order to investigate potential "windows" of heightened sensitivity, embryos were exposed to a range of ethanol concentrations during early, middle or late development and assessed for treatment-related effects on various measures of overt toxicity. Ethanol-induced hatching delays and growth inhibition were used to compare embryonic sensitivity to ethanol toxicity across each exposure window. In general, these results suggest that medaka embryos are sensitive to ethanol toxicity at all stages of development, but demonstrate a variable pattern of effects depending upon the window of exposure.

Abstract

Ethanol is a well-known developmental toxicant that produces a range of abnormal phenotypes in mammalian systems including craniofacial abnormalities, cognitive deficits and growth retardation. Although the toxic potential of developmental ethanol exposure is well characterized clinically, the effect of timing on toxicity remains unknown. Fish models such as the Japanese medaka, *Oryzias latipes*, provide a convenient system for investigating the effects of developmental ethanol exposure *in vivo*. In this study, medaka embryo toxicity tests were used to assess temporal variations in developmental ethanol toxicity. Fertilized eggs were collected and incubated during early, middle or late gestation (*e.g.*, 0-3, 3-6 or 6-9 days post fertilization) with various sub-lethal concentrations of ethanol (0.1%, 0.5%, or 1.0%; *ie*., 17.2, 86, or 172 mM). Time to hatch, head width, and total body length were used to assess toxicity. Hatching delays were noted only at the highest concentration of ethanol, whereas head width was affected at all ethanol levels, regardless of the window of exposure. Ethanol-induced decreases in total body length, however, appeared to be most pronounced when exposure occurred either during the first or last developmental window. Uptake of ethanol by the embryo was ~ 60 to 70% of the nominal (solution) concentration across all windows. In general, these data suggest that critical periods for heightened sensitivity to developmental ethanol exposure vary according to the specific endpoint used to assess toxicity.

Introduction

Women who consume large amounts of ethanol during pregnancy often give birth to children exhibiting phenotypic abnormalities collectively referred to as the Fetal Alcohol Syndrome (FAS). These anomalies include growth deficiency, cognitive impairment and distinctive craniofacial features (Coles 1993). Ethanol is a well-known developmental toxicant in humans and laboratory animals. The primary mechanism for the toxic actions of ethanol on the developing organism is, however, unknown (Goodlett *et al.* 2005). Many hypotheses exist, including interaction with neurotrophins (Kentroti 1997), cell-adhesion molecules (Bearer 2001), or specific receptors (*e.g.,* Costa and Guizzetti 2002); as well as increased apoptosis (Olney *et al.* 2002a; Olney *et al.* 2002b) or oxidative stress (Cohen-Kerem and Koren 2003). Not only are the mechanisms as yet unknown (or multifunctional), but the issue of sensitive periods remains largely unresolved. That is, it is unclear which stage(s) of development is/are most sensitive to ethanol exposure.

Many investigations have used postnatal development of the rat cerebellum to explore temporal variations in developmental ethanol toxicity (Goodlett and Lundahl 1996; Miki *et al.* 1999; Pauli *et al.* 1995; Thomas *et al.* 1998), whereas others have compared late gestational versus early postnatal ethanol exposure (Marcussen *et al.* 1994; Phillips and Cragg 1982). In each case, the Purkinje cells were found to be most sensitive to ethanol toxicity during the first 24 to 48 hours of postnatal development. A slightly different trend was noted in a study of the trigeminal nerve: these neurons were most sensitive to ethanol toxicity during late gestation (when compared to early or late postnatal exposures) (Miller 1995). Very few studies have systematically examined

differences in the vulnerability of the entire central nervous system at distinct periods throughout brain development. There are primate studies, however, that have compared short periods of ethanol exposure early in development with more protracted exposures, which included early and late developmental windows. In most cases the short, early exposure was as detrimental as the longer, more comprehensive exposure (Clarren *et al.* 1992; Schneider *et al.* 2001). A different pattern was noted in a rat study (Tran *et al.* 2000) that compared various windows of ethanol exposure (gestation day 1-10, 11-22 or postnatal day 2-10) using brain weight, brain:body weight ratio, motor activity and exploratory behavior as measures of toxicity. The authors were also careful to equate peak blood ethanol concentrations across all the three exposure periods. In this study, rats exposed to ethanol later in development experienced more adverse effects than those exposed during the earliest developmental window. As yet, the issue of critical windows for developmental ethanol toxicity remains unresolved.

Fish models offer several advantages over traditional mammalian model systems for careful study of developmental timing. Their *ex utero* development eliminates the confounder of maternal toxicity and allows for accurate and precise timing of developmental exposures. Fish are susceptible to a range of developmental toxicities, including that caused by ethanol. Studies of ethanol-induced toxicity in zebrafish have shown that ethanol is a teratogen that produces developmental delays (Reimers *et al.* 2004); craniofacial abnormalities (Bilotta *et al.* 2004; Blader and Strähle 1998; Carvan *et al.* 2004; Loucks and Carvan 2004); skeletal and cardiovascular toxicity (Wang *et al.* 2006a); as well as alterations in eye development (Bilotta *et al.* 2004; Bilotta *et al.* 2002), cell death (Carvan *et al.* 2004; Loucks and Carvan 2004) and behavior (Carvan *et al.*

2004). Because fish are vulnerable to ethanol toxicity and develop *ex utero*, they represent a convenient alternative for exploring temporal variations in ethanol sensitivity during development.

I have examined the interaction between developmental toxicity and the timing of ethanol exposure using the Japanese medaka fish (*Oryzias latipes*). The Japanese medaka, a small, egg laying, freshwater fish native to Japan, Korea, and eastern China, has been widely used in developmental, genetic, and toxicological studies (Gogal *et al.* 1999; Kashiwada *et al.* 2002; Kelly *et al.* 1998; Law 2003; Mullins *et al.* 1994; Wittbrodt *et al.* 2002). Medaka are ideal for developmental studies of the interaction between timing and toxicity when compared to zebrafish because the length of gestation in zebrafish is 3 days, compared to 9 days in medaka. This protracted gestation allows for more accurate delivery of test compounds during discrete periods of development. In the present studies, hatching delays, and growth inhibition were assessed using comparable embryonic ethanol concentrations in order to compare differences in developmental ethanol toxicity following sublethal ethanol exposures during early [0-3 days post fertilization (dpf), middle (3-6 dpf) and late (6-9 dpf) stages of development]. In general, our results suggest that medaka embryos are sensitive to ethanol toxicity at all stages of development, exhibiting a variable pattern of effects depending upon the window of exposure.

Materials and Methods

Test animal, Embryo Collection and Animal Husbandry are as described in Chapter 2.

Chemicals

Ethanol (95%), alcohol dehydrogenase and nicotinamide adenine dinucleotide were purchased from Sigma-Aldrich (St. Louis, MO).

Overt Toxicity (Mortality) Study

Initial range finding studies were performed in order to determine an optimal ethanol dosing regimen for induction of developmental toxicity in the absence of embryo mortality. Embryos (n=16) were exposed to ethanol (0, 0.1, 0.5, 1.0, 1.5, 2.0, or 3.0%, final concentration) (1% ethanol=172 mM) for days 0-8 of gestation, followed by ERM only from days 9 to 15; if an embryo had not hatched by day 15 (99%-100% of the controls hatch by day 15), then the embryo was scored as 'not hatched.' Embryonic death was ascertained as described in Chapter 2. All animal husbandry and plate changing practices were as described in the *Embryo collection and Animal Husbandry* section.

Assessment of the Windows of Vulnerability

Stage 10 embryos were exposed to varying ethanol concentrations [0, 0.1 (17.2 mM), 0.5 (86 mM) or 1.0% (172 mM)] during three distinct periods of development (0-3, 3-6 or 6-9 dpf) (Figs. 3-1; 3-2) in order to investigate the effects of timing and dose on ethanol-induced embryo toxicity. Embryos were assessed for treatment-related effects on growth, mortality and hatching. Morphometric criteria were evaluated on the day of hatching to determine how the timing of ethanol exposure affected medaka growth and development. ERM control replicates were also included as a temporal reference of normal embryonic development.

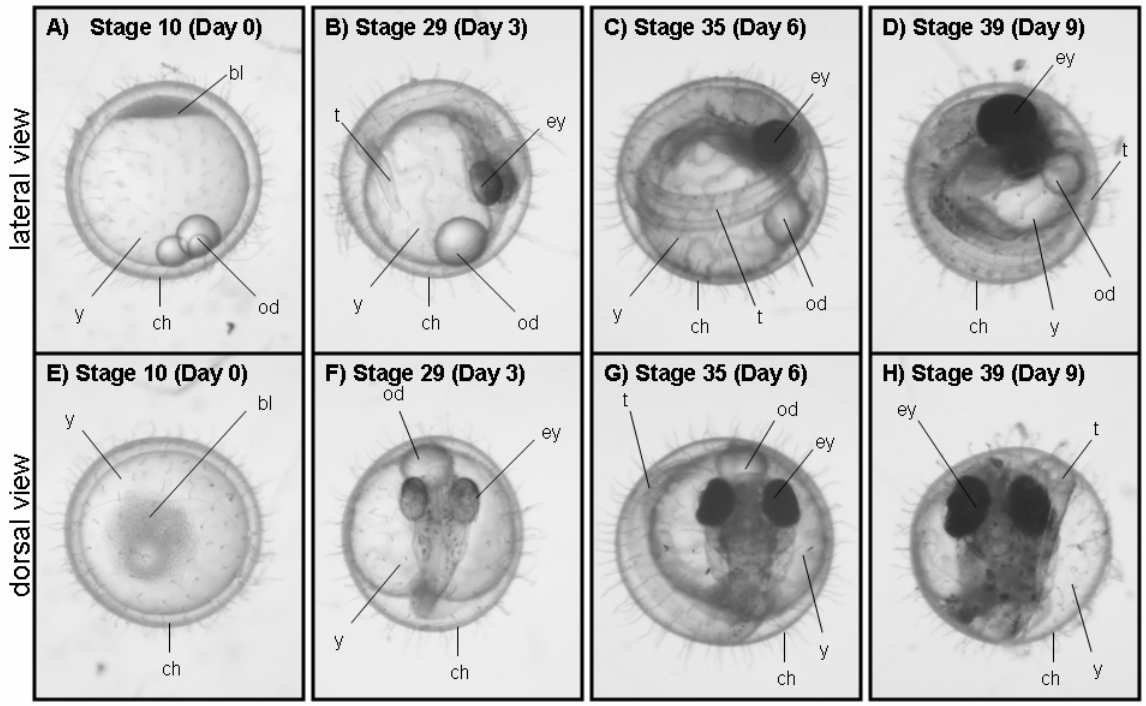
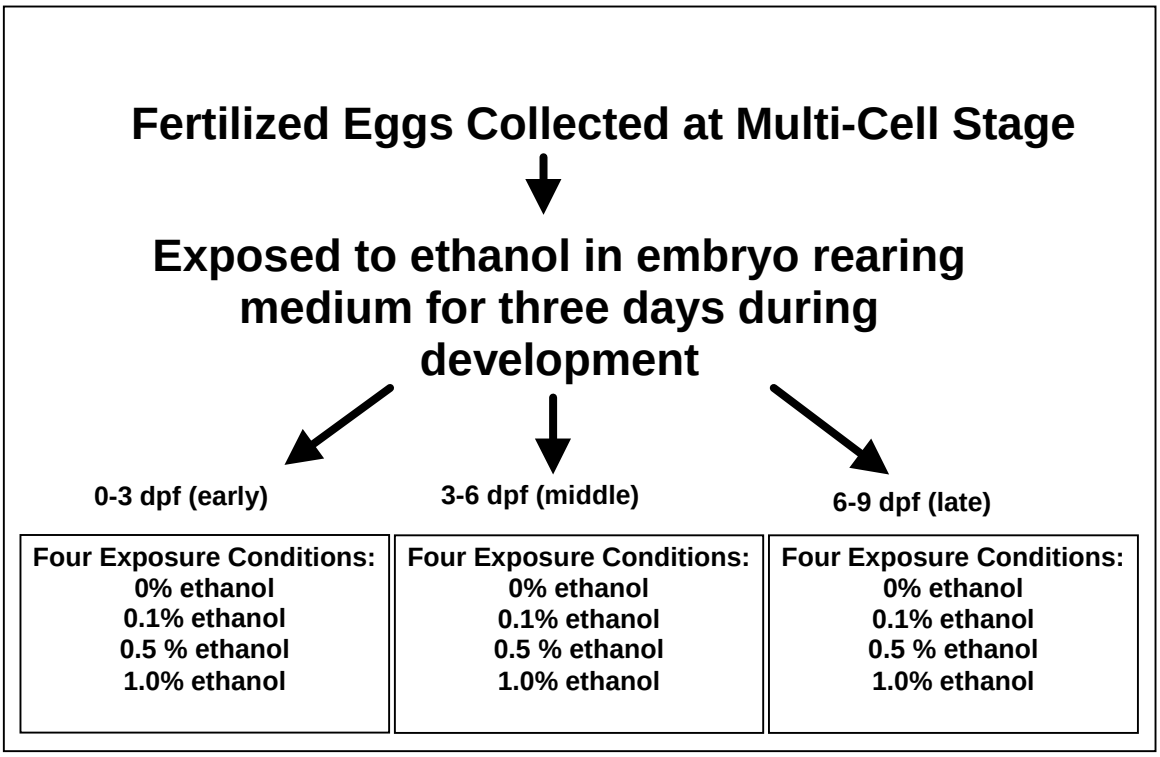


Figure 3-1: Dorsolateral series of figures showing medaka embryonic development. (A) *Multi-cell stage embryo at the early high blastula stage [~6.5 hours post fertilization (hpf); day 0], the animal pole is five cell layers thick, consisting of approximately 1000 blastomeres.* ***(B)*** *Chorionated embryo at stage 29 (~74 hpf; Day 3). The embryo has completed axis formation and somite formation; the eyes are developing.* ***(C)*** *Chorionated embryo at stage 39 (~ 9 dpf; Day 6).* ***(D)*** *By the time embryos are ready to hatch (~Day 9), the tail extends well beyond the posterior head region. At this developmental stage, hatching enzyme released from the buccal cavity breaks down the inner chorion so that embryos may use their body movement to rip through the outer chorion and emerge tail first. Bar = 0.1 mm. bl = blastula, ch = chorion, ey = eye, hpf = hours post fertilization, od = oil droplet, t = tail, y = yolk.*

Figure 3-2: Schematic representation of experimental design*. To investigate the effects of timing and dose on ethanol-induced embryo toxicity, embryos (n=20/dose group/window) were exposed within 96-well microtiter plates to a range of ethanol concentrations (0, 0.1, 0.5 or 1.0%) in embryo rearing medium during three distinct periods of development (e.g., 0-3, 3-6 or 6-9 days post fertilization).*



Morphometric Assessment

Embryos were photographed on the day of hatching using a Nikon SZM 1500 stereomicroscope and coded for a blinded analysis of the effects of developmental ethanol exposure. Linear measures of head width (maximal distance from the lateral aspect of one eye to the most lateral aspect of the other) and total body length (distance from the rostral-most portion of the head to the caudal most portion of the tail fin) were used to

determine whether developmental ethanol exposure induced a phenotype in fish that is qualitatively similar to human fetal alcohol syndrome.

Determination of Embryonic Ethanol Concentration

Ethanol exposed and control embryos were washed three times with ERM, incubated in 3.5% $HClO_4$ (30 embryos per 90 µl solution) for 1hr at 4°C, sonicated on ice and then centrifuged at 2800 x g for 10 min at room temperature. Supernatants were then collected for use in an enzymatic reaction between alcohol dehydrogenase and nicotinamide adenine dinucleotide. NADH accumulation was determined via the method of Reimers *et al.* (2004) with the following modifications to assure linearity with respect to ethanol concentration and incubation time; the concentration of NAD was increased 4x, the concentration of alcohol dehydrogenase was decreased 50%, and the assay was conducted at 30EC instead of 37EC. All assays were performed in 96-well microtiter plates and NADH accumulation was monitored spectrophotometrically using a ThermoMax microplate reader set at 340 nm (Molecular Devices, Sunnyvale, CA). Ethanol standards and sample lysates were read kinetically (at 10 second intervals) for 10 min at 30°C; the slope of the absorbance at 340 nm was used to calculate ethanol concentrations.

Statistical Analyses

All statistical analyses were conducted using StatView® (Version 5.0.1, SAS Institute, Inc.) The two, non-parametric dependent variables (lethality and hatching success) were analyzed using an unpaired, Mann-Whitney test. The parametric data (*i.e.*,

length of time the embryo remained in the egg), were analyzed using a global ANOVA using day of hatch as the dependent variable, and ethanol dose as the independent variable. Any subsequent interactions at the $p \leq 0.05$ level were followed by step-down ANOVAs. When necessary, a Fisher's PLSD post hoc test was conducted to determine differences between ethanol dosage groups. The embryonic ethanol concentration data were analyzed with a repeated ANOVA using embryonic and nominal (solution) ethanol concentration as the dependent variables, and window of exposure as the independent variable.

Results

Overt Toxicity

An initial range-finding study was completed to determine the optimal ethanol dosing regimen for induction of developmental toxicity in the absence of embryo mortality. Ethanol concentrations at or above 2% were lethal to embryos (defined as an opaque egg or the absence of a heartbeat). Embryos exposed to 1% ethanol showed no increased lethality. As the above exposures spanned a period of 8 days, a three day dosing regimen using 1% ethanol as the highest, non-lethal exposure concentration was adopted for subsequent exposures.

Vulnerable Window Studies

Length of Gestation (Time to Hatch): There was no interaction between window of exposure and ethanol treatment. There was, however, an effect of ethanol treatment (Fig. 3-3A) and an effect of window of treatment (Fig. 3-3B). Hatching was delayed by at least

one day in embryos treated with the highest concentration of ethanol (1%) (Fig. 3-3A). This effect was more pronounced in embryos exposed either from 0-3 or 3-6 dpf when compared to embryos exposed from 6-9 dpf.

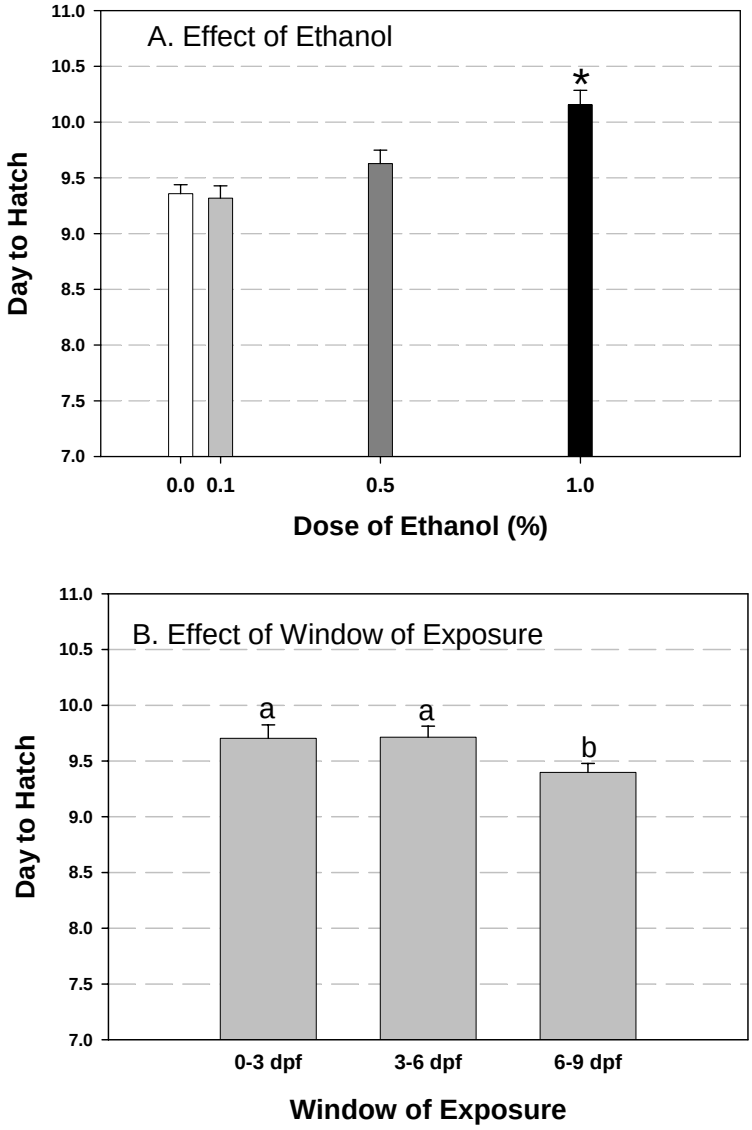


Figure 3-3: Effects of developmental ethanol exposure on time required for hatching. *Values represent the mean ± sem from two cohorts (total n=20/dose group/window). ANOVA analysis revealed that there was no interaction between ethanol dose and window of exposure.* ***Panel A****: These data are collapsed across windows of exposure: only the 1% ethanol exposure delayed hatching.* ***Panel B****: These data are collapsed across all dosage groups. If two bars have the same letter above it, then they are not different from one another. If, however, they have different letters above them, then they are different from one another. In this case, when embryos were treated during the first two windows, there was delayed hatching as compared to no delay if the embryos were treated during the last developmental window. Data are presented as a mean ± sem.*

Head Width: Again, there was no interaction between window of exposure and ethanol treatment. All concentrations of ethanol decreased head width compared to controls, and this effect was not dependent upon the window of exposure (Fig. 3-4).

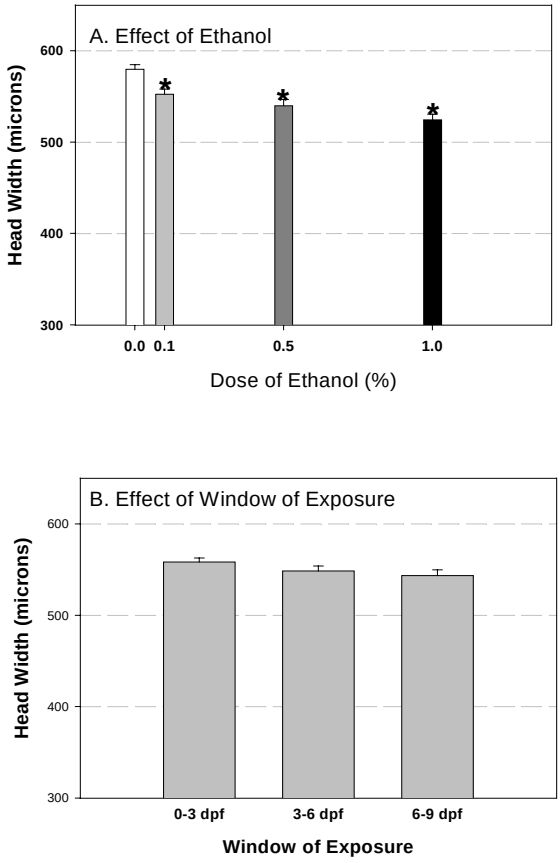


Figure 3-4: Effects of developmental ethanol exposure on head width *in the newly hatched medaka fry. Values represent the mean ± sem from two cohorts (total n=20/dose group/window). Ethanol exposure produced dose-related decreases in head width that were not correlated with the time of exposure. ANOVA analysis revealed that there was no interaction between ethanol dose and window of exposure.* ***Panel A****: These data are collapsed across windows of exposure: all three dose levels of ethanol produced decreased head width.* ***Panel B****: These data are collapsed across all dosage groups. The window of exposure did not affect the decrease in head width. Data are presented as a mean ± sem.*

Body Length: For body length, there was an interaction between ethanol effect and window of exposure (Fig. 3-5). All three ethanol doses decreased body length if embryos were exposed either early (0-3 dpf) or late (6-9 dpf) in development, but this effect was only apparent at the highest (1%) dose when ethanol exposure occurred during the middle window of development (3-6 dpf).

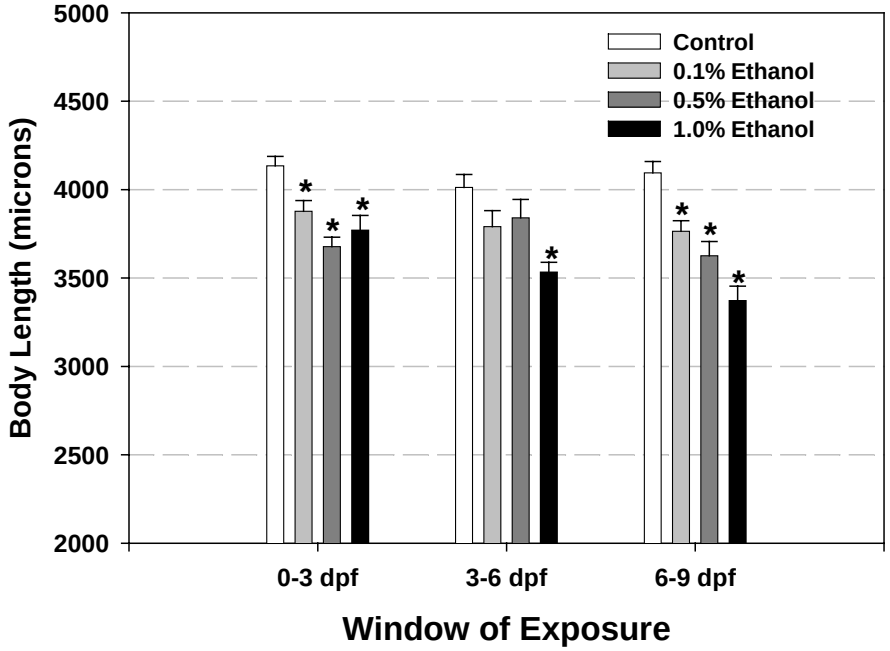


Figure 3-5: Effects of developmental ethanol exposure on total body length *of newly hatched medaka fry. Values represent the mean ± sem from two cohorts (total n=20/dose group/window). Ethanol exposure decreased total body length in a dose- and window-of-exposure-dependent fashion, i.e., there was an interaction between ethanol dose and window of exposure. The degree of ethanol-induced growth inhibition was most severe with high dose exposures that occurred late in development. The asterisks indicate a difference between the ethanol dosed group and the control for each respective window of exposure. Data are presented as a mean ± sem*

Embryonic Concentration of Ethanol During Development:

The above data outline the toxicodynamic effects of ethanol during three windows of development in the medaka fish. In order to determine the concentration of ethanol in

the embryo during these same windows of exposure, embryonic ethanol levels were assessed after exposure to 1% ethanol. Embryos took up roughly the same amount of ethanol regardless of developmental stage (Fig. 3-6).

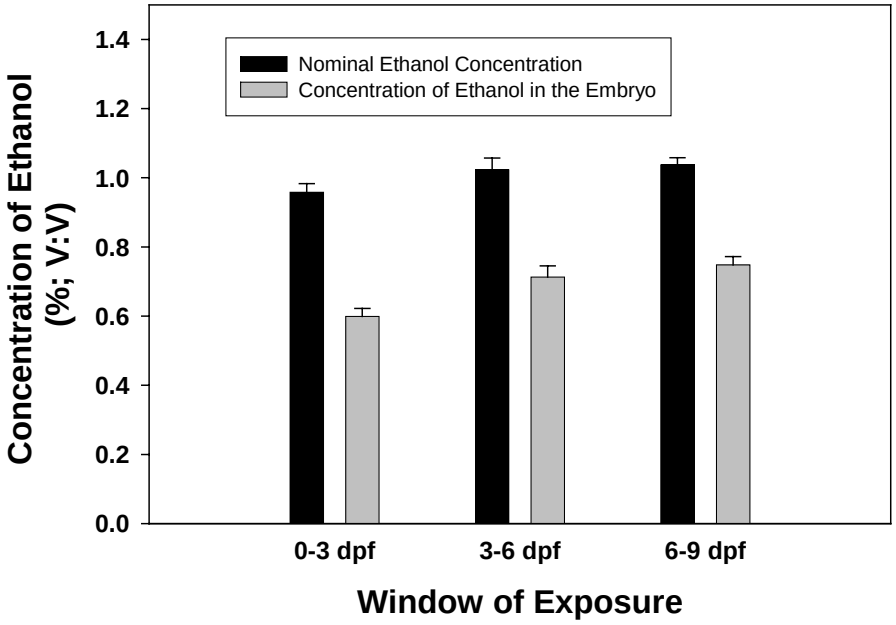


Figure 3-6. Determination of ethanol dose*. The embryonic ethanol dose was assessed after a 3-day exposure to 1% ethanol in embryo rearing medium during early, middle and late development; the solutions were renewed daily. Nominal and embryonic ethanol concentrations were determined (n=3 pools of 30 embryos each). Data are presented as the mean ± sem. Although ANOVA analysis revealed the nominal and embryonic ethanol concentrations to be different from one another, the amount of ethanol taken up by the embryo at each age window was not different depending on the age of the embryo.*

The amount of ethanol that enters the embryo after three days of exposure to 1% ethanol (changing solutions every day) is approximately 60%-70% (grey bars, Fig. 3-6) of the nominal concentration of the ethanol solution contained in the well. Moreover, the concentration present in the solution surrounding the embryo did not decrease from the

original amount (1%) which was added to the well ~24 hours before test samples were taken (black bars in Fig. 3-6).

Discussion

Most FAS studies focus on isolated events that occur within one specific stage of development and/or to one specific group of cells or neurons. Few investigators have examined the global effects incurred when ethanol administration is strictly partitioned within the confines of specific stages of development. The literature seems to suggest multiple effects on multiple systems with no clear indication of whether early or late ethanol exposure is more deleterious to the developing embryo.

The present studies used the extended duration of medaka embryonic development to assess temporal variations in developmental ethanol toxicity. Table 3-1 shows that, in general, no developmental window is the least or most sensitive, rather the pattern of effects depends upon the endpoint examined. If ethanol exposure occurred early in development (0-3 dpf), embryos were extremely sensitive to reduced head width and total body length. A similar pattern was observed when the embryos were exposed late in development (6-9 dpf). Embryos exposed to ethanol during the middle interval of development (3-6 dpf), however, appeared less responsive to ethanol-induced decreases in total body length and equally responsive to ethanol-induced decreases in head width. Interestingly, some measures, (*e.g.*, head width), are changed at all doses and all windows of exposure, whereas others (*e.g.*, total body length) are highly sensitive to the window of exposure.

Endpoint	Window of Exposure		
	0-3 dpf	3-6 dpf	6-9 dpf
Gestational Length (Hatching)	↑ only at highest ethanol dose	↑ only at highest ethanol dose	No effect
Head Width	↓ at all doses	↓ at all doses	↓at all doses
Body Length	↓ at all doses	↓ only at highest ethanol dose	↓at all doses
Embryonic [Ethanol]	60% of nominal	66% of nominal	69% of nominal

Table 3-1: Summary of Data from Chapter 3*. Data presented in the grey shaded region is from animals treated only with 1% ethanol.*

To put these changes in perspective, one can compare the results in Table 3-1 with known effects of ethanol on mammalian development. In humans, ethanol exposure during gestation produces persistent decreases in height and head circumference (Habbick *et al.* 1998; Sokol and Clarren 1989). These parameters are generally considered to be the most sensitive diagnostics for ethanol-induced intrauterine growth restriction (Streissguth *et al.* 1991). This treatment effect was also observed in the present study, which showed ethanol–induced deficits in total body length and head width even at the lowest ethanol concentration (0.1%; nominal).

Comparison of the present data on ethanol toxicity in medaka with previously published studies of ethanol toxicity in fish shows some similarities and some differences. The dose of ethanol producing overt toxicity (*i.e.*, 2% ethanol) is very similar to that noted in other studies. Wang and coworkers estimated the 48 hour LC_{50} for ethanol exposure in medaka embryos to be ~3.2% (Wang *et al.* 2006a), which is comparable to the present lethal dose calculation of ~2% for an 8 day exposure. The zebrafish literature indicates that sensitivity to ethanol lethality is strain dependent (Loucks and Carvan 2004), with the three day (*i.e.*, embryonic) LC_{50} hovering around 1.7 to 2.0% (Loucks and Carvan 2004; Reimers *et al.* 2004). There is disagreement, however, regarding how much ethanol enters the embryo. Although Blader and Strähle (1998) assumed that 100% of the nominal (*i.e.*, media) ethanol concentration reached the zebrafish embryo because the rate of cyclopia was the same both in chorionated and dechorionated embryos, later assessments of the intra-embryonic ethanol dose was estimated to be only 30% of the nominal ethanol concentration in zebrafish (Reimers *et al.* 2004) or 20% of the nominal ethanol concentration in medaka (Wang *et al.* 2006a). These findings are contrasted by the present data which indicate that approximately 60-70% of the waterborne ethanol concentration enters the embryo. These differences could be due to the longer exposure interval (3 days) used in the present study or to our optimization of the enzymatic assay used for ethanol determinations.

Central nervous system structures are heterogeneous, with individual neurons developing on different timescales that vary considerably across brain regions and neuronal populations. If the mechanism of ethanol toxicity is not specific for the type of neuron affected, but instead dependent upon the precise stage of neuronal development,

this may explain the lack of specificity observed with ethanol related developmental effects. The more homogenous an experimental system is, the more likely a critical window for ethanol toxicity may be identified, but as the system becomes more heterogeneous (as in the entire brain), the temporal effects of ethanol toxicity are going to appear multifaceted and diffuse.

In his comprehensive review of critical windows for developmental ethanol toxicity, (West 1987) concluded that the human third trimester equivalent appeared to be most sensitive to developmental ethanol toxicity. That conclusion is confounded by (1) the lack of standardization with regard to blood ethanol concentrations and (2) the lack of available information regarding temporal sensitivity, as very few investigators have compared the differential effects of ethanol toxicity when peak blood ethanol concentrations are equated across all three trimesters of development. In fact, West later goes on to suggest that the human third trimester equivalent may simply *appear* more sensitive to developmental ethanol toxicity because, in general, the blood ethanol concentrations tended to be higher when animals were exposed during this developmental window. In the present study, I have addressed many of these issues by conducting a side by side comparison of different developmental windows while maintaining a comparable ethanol body burden in the embryo. Our general conclusion echoes the conclusions of (West 1987) and (Tran *et al.* 2000); ethanol is toxic at all developmental windows, but if a single window must be chosen, then the last exposure window appears more sensitive.

CHAPTER 4.

EFFECT OF ETHANOL ON THE DEVELOPMENT OF THE NERVOUS SYSTEM OF THE MEDAKA: WINDOWS OF SENSITIVITY

Preface

Work described in this chapter relates primarily to Aim 3. Medaka embryos exhibited dose-related decreases in head width and brain volume as a result of developmental ethanol exposure. Ethanol-induced increases in programmed cell death have been implicated in microencephaly associated with FAS, therefore, these experiments specifically addressed caspase-3/7 activation as a potential mechanism of ethanol-related decreases in brain growth. Although ethanol-induced decreases in brain volume were most pronounced with early or late developmental exposures, treatment-related changes in caspase-3/7 activation did not vary with the timing of exposure. These studies revealed that developmental ethanol exposure inhibits normal increases in caspase-3/7 activation that occur naturally during development, indicating that increased cell death may not be the primary mechanism for ethanol-induced decreases in brain size.

Abstract

Ethanol-related defects in CNS morphogenesis may precipitate a number of teratogenic events, including microencephaly and mental retardation. Ethanol-induced increases in programmed cell death (apoptosis) may contribute to CNS insufficiency. In this study, medaka embryo toxicity tests were used to investigate temporal changes in ethanol-induced activation of the apoptotic executor, caspase-3/7. Two exposure paradigms were used for this evaluation. In the first cohort, control and 1% ethanol treated embryos were assessed for caspase-3/7 activation sequentially (*e.g.*, every 24 hrs) throughout gestation. In the second cohort, embryos were exposed to 1% ethanol during early (0-3 dpf), middle (3-6 dpf) or late (6-9 dpf) gestation and assessed for changes in caspase-3/7 activation on day 9 post fertilization. Increases in caspase-3/7 activation were noted in embryos treated on days 1 or 2 (early), whereas decreases were seen in embryos treated on day 6 (middle) or day 8 (late). When the duration of exposure was increased to 72 hrs and embryos were subsequently assessed on day 9, there was a substantial effect of ethanol exposure, but no effect of developmental timing. Marked decreases in caspase-3/7 activity were observed for all windows of exposure. Ethanol appeared to blunt the increase in caspase-3/7 activity that occurs naturally during embryonic development, suggesting that developmental ethanol exposure may not exert its morphogenic effects via increased apoptosis.

Introduction

Although dysmorphic effects are commonly seen in FAS, ethanol-induced changes in CNS development may occur without concomitant deformity. "Alcohol-related neurodevelopmental disorders" (ARND) is a term used to describe individuals who experience mental and physical impairments as a result of prenatal ethanol exposure (in the absence of craniofacial malformations). These neurodevelopmental effects are often as severe as those seen with full blown FAS (Mattson *et al.* 1994).

There is no doubt that ethanol adversely affects brain development. Humans and experimental animals exposed to ethanol during gestation exhibit permanent deficits in CNS structure. Post mortem (Clarren 1981; Clarren *et al.* 1978; Peiffer *et al.* 1979) and brain imaging [(Mattson *et al.* 1996; Ronen and Andrews 1991); and reviewed in Roebuck *et al.* 1998] studies have confirmed malformations in brains of children exposed to ethanol *in utero*. Microencephaly was the most consistent finding, with FAS patients showing substantial reductions in brain volume relative to controls (Archibald *et al.* 2001; Sowell *et al.* 1996). Rodent models have also been valuable for demonstrating region-specific manifestations of developmental ethanol toxicity *in vivo* (Kotch and Sulik 1992; Webster *et al.* 1983; West 1987). Animal studies have unveiled a number of forebrain deficiencies following prenatal ethanol exposure (Ashwell and Zhang 1994; Maier *et al.* 1999a; Maier *et al.* 1999b; Webster *et al.* 1980).

Very few investigators have compared ethanol-induced changes in brain volume or brain weight during discrete periods that encompass early, middle or late development. The most comprehensive and well-controlled study with regard to BEC was conducted 6 years ago by Tran and coworkers (Tran *et al.* 2000). They found that rats exposed to

ethanol during GD 11-22 or PD 2-10 experienced decreases in brain weight, whereas those exposed during the earliest developmental window (GD 1-10) did not. As depressed brain volume/weight appears to be a common result of developmental ethanol exposure in humans, and laboratory animals exhibit regional differences in ethanol-induced inhibition of brain growth, I wanted to determine (1) if developmental ethanol exposure decreases brain volume in medaka embryos; (2) if temporal variations in ethanol-induced inhibition of brain growth coincide with a specific exposure window and (3) if treatment-related changes in brain volume correlate with changes in the apoptotic indicator, caspase-3/7. Temporal variations in ethanol-induced inhibition of somatic growth were demonstrated in Chapter 3. This chapter investigated a neurodevelopmental endpoint that is known to be affected by developmental ethanol exposure. Specifically, ethanol-induced changes in brain growth were assessed in order to determine whether medaka embryos experience spatiotemporal variations in developmental ethanol toxicity.

Materials and Methods

Chemicals

Chemicals were as described in Chapter 3

Test animal

Japanese medaka embryos used in this study were collected from an orange-red line maintained under standard re-circulating aquaculture conditions. See Chapter 3 for a detailed description.

Research design

Research design was as described in Chapter 3 except that the embryos were exposed to either 0 or 1% ethanol during different windows of development. The lower concentrations of ethanol were not used.

Fixation and Histological Procedures

On the day of hatching, control and treated fry were anesthetized in ice-cold ERM and fixed in 4% paraformaldehyde/phosphate buffered saline (PBS; pH 7.4) for 48 hrs at 4°C. Specimen were stored in 6% sucrose/PBS (pH 7.4) at 4°C until mounting and sectioning. Hatched fry were oriented vertically with the rostral portion down and double embedded in paraffin. Each block of paraffin contained a control and a treated animal from each exposure window. Coronal step sections (5 μm thickness) taken throughout the brain were then mounted on glass slides and stained with hematoxylin and eosin for neuroanatomical assessment. In practice, approximately 70-80 sections were sufficient to image the forebrain, midbrain and rostral portions of the hindbrain. Coronal sections were terminated at the point where separation between ventral and dorsal hindbrain regions was no longer apparent. The intestinal bulb and liver was also visible in these sections. Images were captured with a Nikon Eclipse TE 200 stereomicroscope, and Spot Advanced imaging software.

Estimates of Brain Volumes

All hematoxylin and eosin stained sections were photographed using a 20x objective and printed. The area of each brain section was estimated using the Cavalieri

method (Howard and Reed 1998). Briefly, a 15 x 20 grid was randomly placed over the section and all the intersections that fell within the forebrain were counted. The volume of the brain was calculated as the sum of the areas of the sections (taking into consideration the size of the grid and the magnification) times the thickness of the sections, assuming a 5 micron nominal thickness of each section. In cases of missing sections, the volume of the absent region was estimated from adjacent sections using the formula for the volume of a truncated cone:

$$V = (5 * NMS) * \frac{A_{Adj1} + A_{adj2}}{2}$$

NMS = number of missing sections

A_{Adj1} = area of one adjacent section

A_{Adj1} = area of the other adjacent section.

To ensure consistency in determining the rostral-caudal boundaries for volume estimates, landmarks were used (Anken and Bourrat 1998). The rostral brain landmark was the ventral telencephalon; the caudal landmark of the brain was the caudal extent of the inferior lobule. The volume of the rostral and caudal halves of each brain were calculated as the brain volume of the first half of the sections and the volume of the caudal half was calculated from the caudal half of the sections.

Caspase Activity

Embryos at each age were treated with 1% ethanol for 24 hours. At the end of this exposure period, groups of embryos were homogenized (n=6 pools of 10 embryos at each age; 10 embryos per 100µL) in lysis buffer (10 mM HEPES, 42 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreotol, 0.1 mM EDTA, 0.1 mM EGTA; pH 7.4) and centrifuged at 2800 x g for 20 min at 4°C. Supernatants were aliquoted and stored at -80°C until analysis. Caspase activation was measured as the release of aminoluciferin from a selective substrate for caspase-3/7 (Z-DEVD-AML) using the Caspase-Glo 3/7® Luminescence Assay kit (Promega, Madison, WI). Luminescence was determined using a FLUOstar Optima luminometer. The assay was optimized so that caspase-3/7 activity was linear with respect to protein concentration and incubation time. On the day of assessment, all samples were thawed on ice and 10 µl of the sample lysate (~30 µg protein) was added to 90µl of the Caspase-Glo substrate and incubated in the dark for 1 hr at room temperature. Purified caspase-3 enzyme (Biomol #SE-169, Plymouth Meeting, PA.) was included as a positive control, and addition of the specific caspase-3 inhibitor, AC-DEVD-CHO (Calbiochem) was used to show that approximately 90% of the observed luminescence was due to caspase-3 activity (data not shown).

Statistical Analyses

All statistical analyses were done using StatView® (version 5.0.1), and all data are presented as mean ± standard error of the mean. The brain volumes were first compared using a global (repeated) ANOVA with total, rostral and caudal brain volume as the dependent variables and treatment (ethanol or control) or window (control, 0-3, 3-

6, or 6-9 dpf) as the independent variables. If the interaction between endpoint and treatment or window of treatment was $p \leq 0.10$, then a step down ANOVA was performed for each type of volume estimate. If that ANOVA was significant at the $p \leq 0.05$ level, then a Fisher's PLSD *post hoc* test was conducted to determine which window of treatment was different from the controls.

Caspase data collected during embryogenesis were analyzed using a global ANOVA with caspase activity as the dependent variable and age of the embryo and ethanol exposure as the independent variables. A Fisher's PLSD post hoc test was conducted to determine differences between the ethanol treated and control groups. The caspase data assessed on day 9 were first compared by ANOVA to determine if there was an overall effect of treatment: ethanol treated vs. control groups. If the $p \leq 0.10$, then a second, step-down ANOVA was conducted to determine if there was an effect of window of treatment, using window of treatment as the independent variable and caspase activity at day 9 as the dependent variable. If the $p \leq 0.05$, then between groups comparisions were conducted using a Fisher's PLSD test.

Results

Figures 4-1 and 4-2 show coronal sections of the newly hatched fry brain at two different anatomical levels. The optic chiasm level (Fig. 4-1) is representative of the rostral brain and the optic tectum level (Fig. 4-2) is representative of the caudal mid-brain. Each figure shows representative sections from all treatment groups. When comparing coronal brain sections it was very difficult to identify specific treatment-related effects due to variability in section orientation. Assessments were further

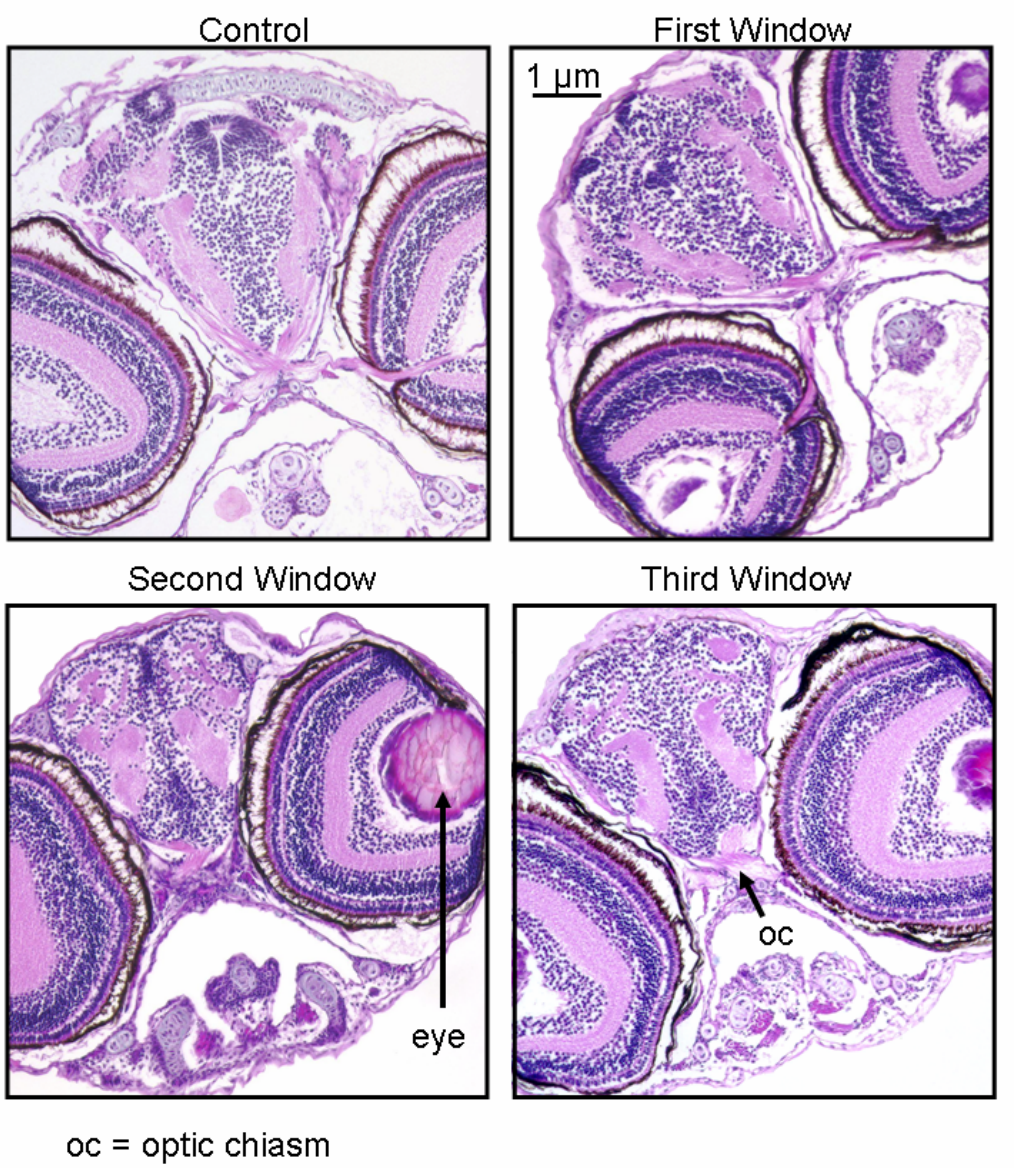


Figure 4-1: Coronal Sections of the Rostral Brain in the Medaka Fry at the Level of the Optic Chiasm. *Medaka were treated with 1% ethanol during the three windows of development (0-3 dpf, 3-6 dpf, or 6-9 dpf). On the day of hatching (usually day 10), the medaka were fixed and later embedded in paraffin for histological assessment.*

complicated by the fact that there is no atlas for the medaka fry brain, requiring that many structures be inferred using the atlas for the adult medaka brain (Anken and Bourrat 1998). As examination of individual sections did not provide any consistent treatment-related patterns, I then used these sections to calculate total brain volume, which ranged from ~150,000 – 300,000 cubic microns in treated and control animals, respectively.

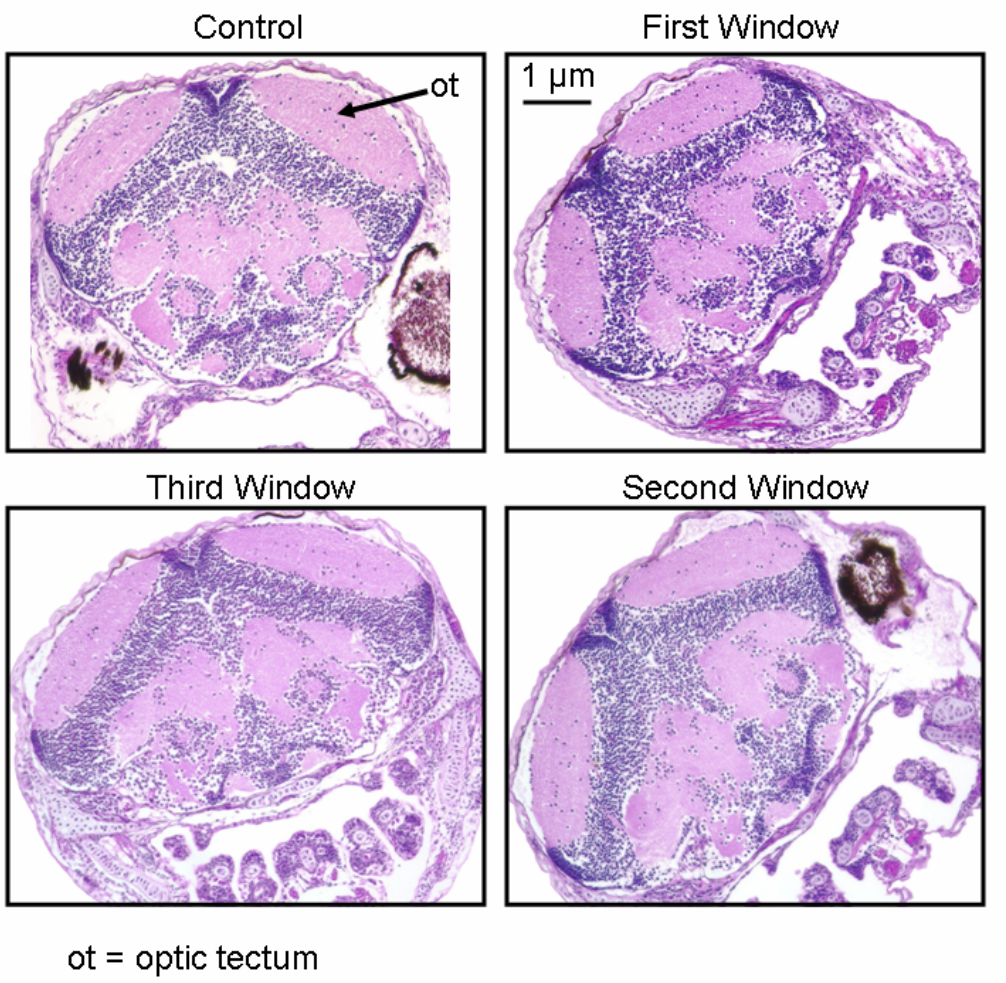


Figure 4-2: Coronal Sections of the Caudal Mid-Brain in the Medaka Fry at the Level of the Optic Tectum*. Medaka embryos were treated with 1% ethanol during the three windows of development (0-3 dpf, 3-6 dpf, or 6-9 dpf). On the day of hatching (usually day 10), the medaka were fixed and later embedded in paraffin for histological assessment.*

Treatment with 1% ethanol during different windows of gestation produced profound effects on brain volume in the newly-hatched fry (Figs. 4-3 and 4-4), and observed decreases in brain volume were dependent upon both the window of exposure (Fig. 4-3) and the area of the brain being assessed (Fig. 4-4). Medaka embryos treated with 1% ethanol during early (0-3dpf) or late (6-9 dpf) development experienced decreases in total brain volume (Fig. 4-3), whereas embryos treated during mid-gestation (3-6 dpf) did not. Interestingly, the effect of ethanol was much more pronounced in the rostral portion of the brain (Fig. 4-4) when compared to the caudal portion (Fig. 4-4) of the brain. In the rostral portion of the brain, the pattern of effect mimicked what was seen in the entire brain (Fig. 4-3) with the first and last exposure windows showing decreased brain volume and the middle exposure window showing no effect. This is contrasted by the pattern of effect noted in the caudal portion of the brain, where animals treated during the first window (0-3 dpf) showed an effect on brain volume, whereas animals treated during the second (3-6 dpf) or third (6-9 dpf) developmental windows did not.

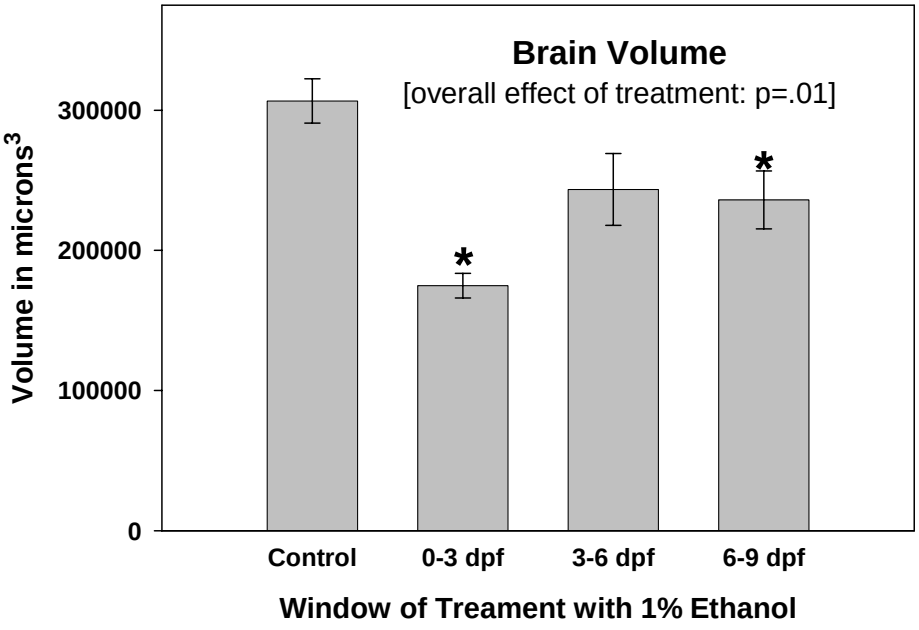


Figure 4-3: Comparison of the brain volume of the medaka fry treated with ethanol during the three windows of development*. Medaka were treated with 1% ethanol during the three windows of development (0-3 dpf, 3-6 dpf, or 6-9 dpf). On the day of hatch (usually day 10), the medaka were fixed and later embedded in paraffin for histological assessment. Volume was calculated from areal point counts using stereological techniques. An overall ANOVA indicated that there was a main effect of ethanol treatment (see figure), and an effect of window of treatment. * indicates that the ethanol treated group is different from the control group ($p \leq 0.05$). Data are presented as the mean ± standard error of the mean. n=4, except for the 0-3 dpf treated embryos where the n=2*

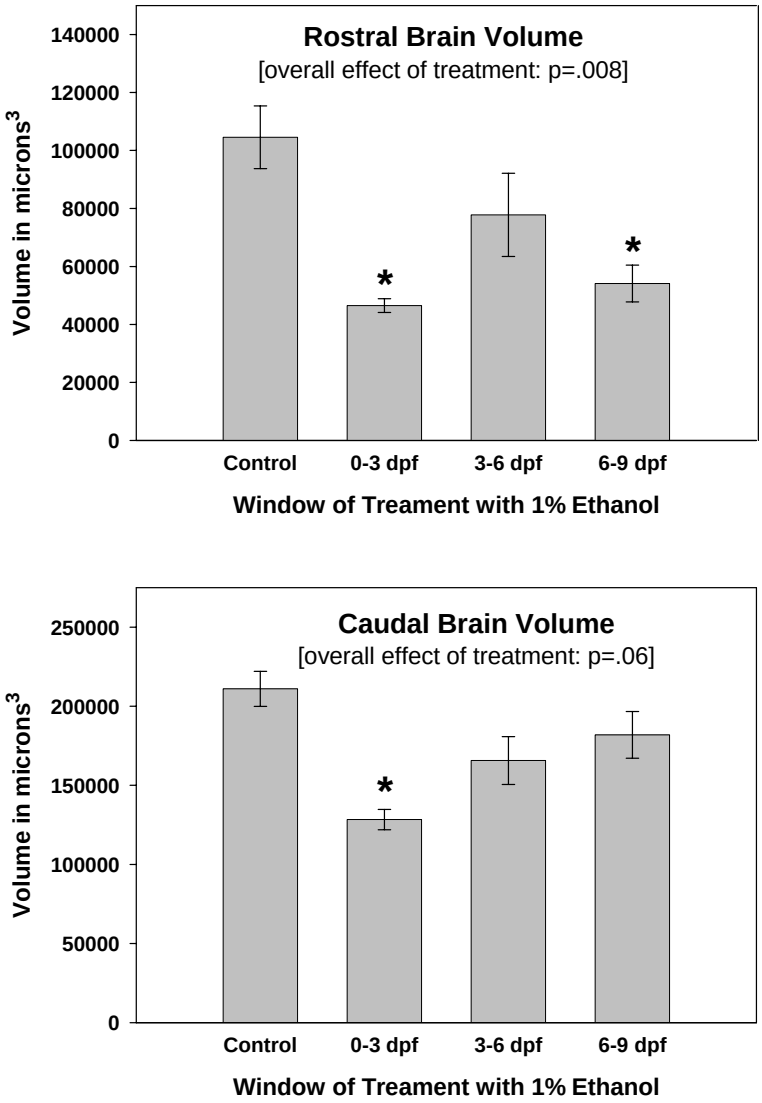


Figure 4-4: Comparison of the rostral and caudal half of brain volume in medaka fry treated with ethanol during the three windows of development*. Medaka were treated with 1% ethanol during the three windows of development (0-3 dpf, 3-6 dpf, and 6-9 dpf). On the day of hatch (usually day 10), the medaka were fixed and later embedded in paraffin for histological assessment. Volume was calculated using stereological techniques. An overall ANOVA indicated that there was a main effect of ethanol treatment, and an effect of window of treatment. * indicates that the ethanol treated group is different from the control group ($p \leq 0.05$). Data are presented as the mean ± standard error of the mean. n=4, except for the 0-3 dpf treated embryos where the n=2.*

Caspase-3/7 Activity

Under normal conditions, caspase-3/7 activity in the medaka embryo increases dramatically within the first seven days of embryonic development, exhibiting an 18 fold increase in activity between days 1 and 9 post fertilization (Fig. 4-5A). Interestingly, there were two plateaus in caspase-3/7 activity that occurred between 3-4 dpf and then again between 6-7 dpf (Fig.4-5A). During the first three days of development, caspase-3/7 activity increased approximately 2-fold, and then increased more than 3-fold between 4-6 dpf. This was followed by a 2.5 fold increase in caspase-3/7 activity that occurred during the last three days of development (6-9 dpf) (Fig. 4-5A). A brief, 24 hour treatment with 1% ethanol produced differential effects on caspase-3/7 activity depending upon the timing of exposure; data are presented in blocks of three days due to differences in the magnitude (scale) of caspase activity. For example, if embryos were treated with ethanol on days 1 or 2 post fertilization, there was an increase in caspase-3/7 activity (Fig. 4-5B); however if embryos were treated with 1% ethanol on day 6 post fertilization, there was a substantial decrease in caspase-3/7 activity (Fig. 4-5C), and if embryos were treated on day 8 post fertilization, a more attenuated decrease in caspase 3/7 activity was observed (Fig. 4-5D).

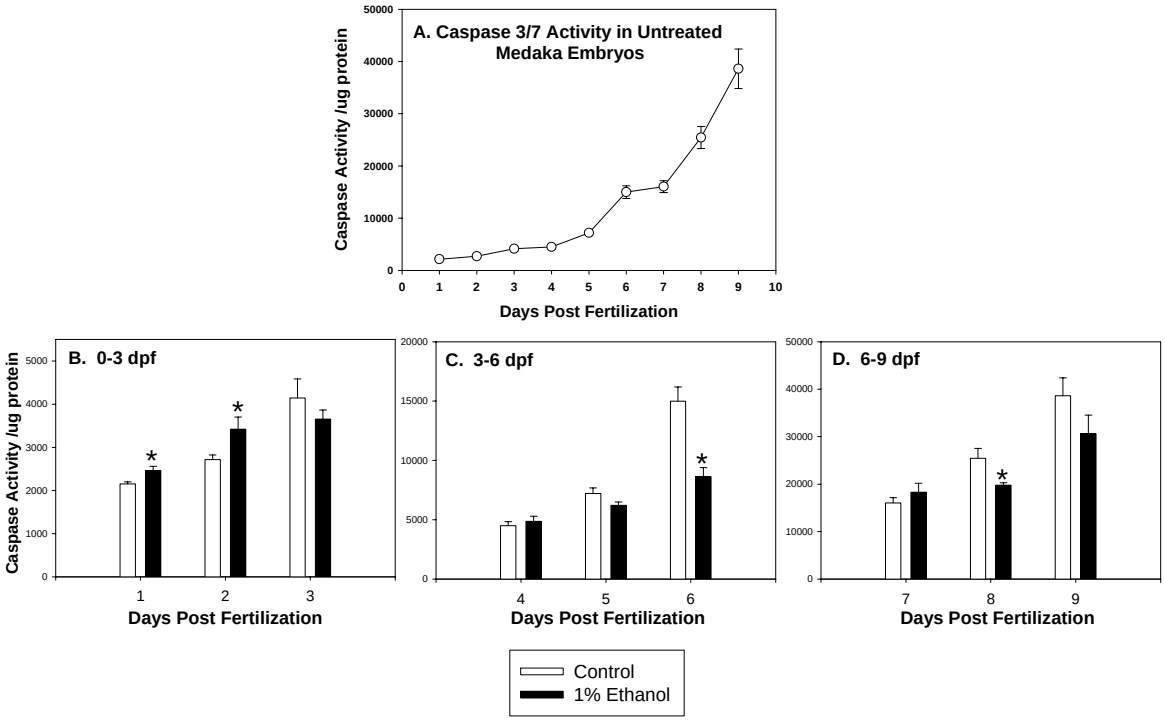


Figure 4-5: Caspase-3/7 activity in the developing medaka embryo *(upper panel) and the effects of developmental ethanol exposure on caspase-3/7 activity (lower three panels; note that the y axes are different on the lower three panels). Values represent the mean response from three cohorts (total n=6; each observation was a pool of 10 embryos). A single 24 hr exposure to 1% ethanol induced reciprocal effects on caspase-3/7 activity depending upon the timing of exposure. Early developmental ethanol exposure increased and later developmental ethanol exposure decreased caspase-3/7 activity. Data are presented as a mean ± sem. An overall ANOVA indicated that there was a main effect of ethanol treatment and a main effect of age of the embryos and an interaction between the two. Step-down ANOVAs comparing the caspase-3/7 activity for each age embryo revealed differences at days 1, 2, 6 and 8 post fertilization. * indicates that the ethanol treated group is different from the age-matched control (p≤ 0.05). The control data in the upper panel and the lower panels are identical.*

Treatment with 1% ethanol for three days during the first (0-3 dpf), second (3-6 dpf) or third (6-9 dpf) developmental window with an assessment of whole embryo caspase activity on day 9 produced a different pattern of effect. In this case, there was a

marked decrease in caspase activity in treated embryos relative to controls, regardless of the window of exposure (Fig. 4-6).

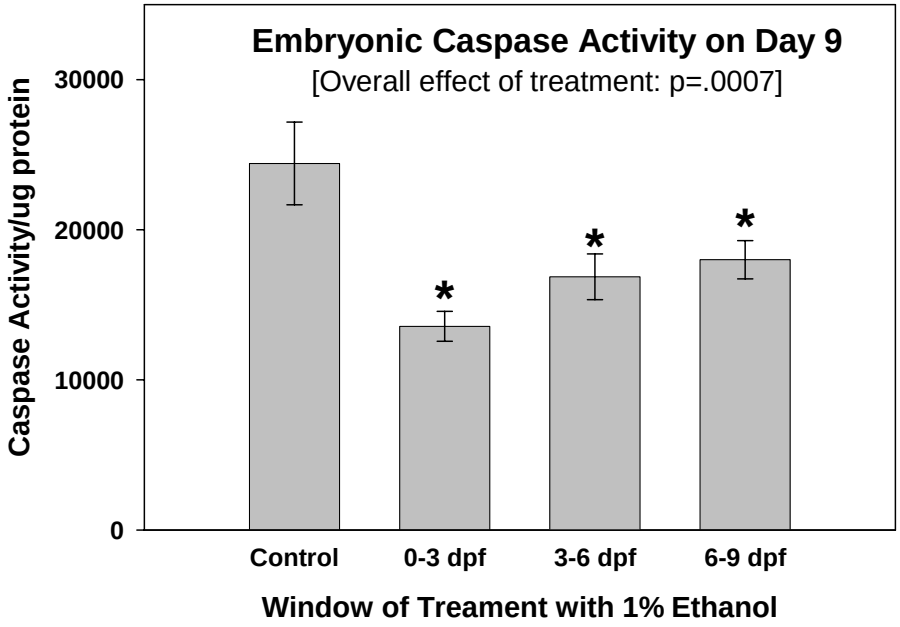


Figure 4-6: Caspase-3/7 activity in the medaka embryo on Day 9 of development*, right before hatching. Each group of embryos was treated with 1% ethanol during the three windows of embryonic development: first (0-3 dpf), middle (3-6 dpf) or late (6-9 dpf). Then on day 9 post fertilization, whole body caspase-3/7 activity was assessed. Ethanol treatment during all windows produced a profound decrease in caspase-3/7 activity. An overall ANOVA indicated that there was a main effect of ethanol treatment, but no window x treatment interaction. * indicates that the ethanol treated group is different from the control group (p≤ 0.05). Data are presented as a mean ± sem. Values represent the mean response from n=6; each observation was a pool of 10 embryos*

Discussion

The literature shows that developmental ethanol exposure produces permanent changes in brain structure and differentially affects specific cell populations and brain regions, but information regarding the spatiotemporal effects of developmental ethanol

exposure is sparse, both in humans and in experimental animals. Research presented in this chapter addressed the relationship between timing and toxicity by examining spatiotemporal variations in the observed manifestations of developmental ethanol toxicity in medaka fish.

Our primary objective was to determine whether developmental ethanol exposure produced decreases in medaka brain size, as previously noted in humans and experimental animals. The second objective of these studies was to identify the most sensitive period for ethanol-induced decreases in brain volume. These results indicate that embryonic exposure to 1% ethanol during discrete periods of development causes profound decreases in brain volume (Fig 4-3). Specifically, developmental ethanol exposure produced decrements in brain growth that were most severe with treatment occurring either during early or late development (Fig. 4-3). Although these findings are consistent with a number of reports indicating ethanol-induced decreases in total brain volume/weight (Archibald *et al.* 2001; Ashwell and Zhang 1994; Chen *et al.* 1998; Maier *et al.* 1999a; Maier *et al.* 1999b; Sowell *et al.* 1996), they differ somewhat from those of Tran and coworkers, who observed marked inhibitory effects on brain weight with middle or late, but not early ethanol exposure (Tran *et al.* 2000). This disparity may reflect procedural differences, as this research group used an ethanol dose that was approximately 50% of that used in the present study. Developmental ethanol exposure also exerted region-specific effects on embryonic brain growth (Fig. 4-3). Substantial decreases in total brain volume were observed in treated embryos relative to controls with a disproportionate reduction in rostral brain regions (Fig. 4-4). This finding is consistent with previous reports of hypoplastic forebrain development in humans (Mattson *et al.*

1994), as well as mouse (Ashwell and Zhang 1994) and avian (Cartwright and Smith 1995) models of FAS.

In contrast to the enhanced sensitivity observed with early or late ethanol exposure, embryos treated during the middle developmental window appeared less responsive to developmental ethanol toxicity. This treatment group was relatively resistant to ethanol-induced decreases in brain volume and structural integrity. A similar pattern of ethanol sensitivity was described in Chapter 3, wherein embryos treated during 3-6 dpf were less affected by ethanol-induced decreases in somatic growth (Table 3-1) than those treated during early or late development. At this point, it is unclear why this treatment group remained largely unaffected by developmental ethanol exposure, but it is possible that temporal differences in gene expression may confer susceptibility or resistance to ethanol toxicity at various stages of development (Dunty *et al.* 2001). Regional differences in patterning genes that are essential for morphogenesis, including members of the Hox family (Trainor and Krumlauf 2000), may influence spatiotemporal aspects of developmental ethanol toxicity. Phenotypic similarities between ethanol-exposed and Hox $^{-/-}$ embryos suggest that these genes may be selectively targeted by ethanol (Barrow *et al.* 2000). In addition, (Dupe and Lumsden 2001) have shown that the dependency on signaling morphogens is progressively lost in a rostral-to-caudal fashion as neurodevelopment proceeds. This could partially explain the enhanced sensitivity observed with early ethanol exposure, but does not account for the relative insensitivity observed with later exposures.

No single mechanism can account for all of the adverse effects observed in FAS, but animal models have identified several possibilities, many of which precipitate cell

death by apoptosis or necrosis. These include ischemia, oxidative stress and subsequent mitochondrial damage, interference with growth factor and second messenger function, decreased protein synthesis, insufficient nutrient utilization, abnormal glucose metabolism, altered prostaglandin homeostasis, depletion of energy stores and aberrant cell cycle kinetics (Abel and Hannigan 1995; Guerri *et al.* 1984; Maier *et al.* 1996; Phillips and Cragg 1982; Santillano *et al.* 2005; Schenker *et al.* 1990; West *et al.* 1994). Our initial data seemed to support changes in apoptosis as a potential mechanism of developmental ethanol toxicity. When embryos were treated with 1% ethanol and caspase-3/7 activity was measured after 24 hours of exposure, there were changes in caspase-3/7 activity regardless of the window of exposure. Increased caspase activation was noted if embryos were exposed to ethanol on days 1 or 2 of development, however, if the same exposure occurred on day 6 or day 8, substantial decreases in caspase-3/7 activity were noted. Interestingly, when embryos were exposed to ethanol for 72 hours during early, middle or late development and caspase-3/7 activity was subsequently assessed on day 9 (immediately before hatching), all three treatment groups showed marked decreases in caspase-3/7 activity regardless of the window of exposure. Although an increase in caspase-3/7 activity was expected (indicatng treatment-related increases in apoptosis), there was actually a decrease in whole embryo caspase activity following developmental ethanol exposure, suggesting that apoptotic cell death may not be the primary mechanism for ethanol-induced growth retardation in this particular model.

While it is generally well-accepted that an increase in caspase-3/7 activity signifies an increase in apoptosis, it is becoming increasingly clear that this view may be too simplistic. For example, caspase-3/7 can exert non-apoptotic effects on cellular

differentiation [(Ishizaki *et al.* 1998); for recent reviews see Abraham and Shaham 2004; Launay *et al.* 2005; Sehra and Dent 2006]. Our results are consistent with a recent report of decreased caspase-3 activity in rat pancreas following developmental ethanol exposure (Wang *et al.* 2006b). Although changes in caspase-3 activity are often viewed in terms of apoptotic potential, an alternative explanation for treatment-related changes in enzyme activity could be dysregulation of the developmental differentiation profile of the embryo. As mentioned above, the developmental effects of ethanol exposure include changes in differentiation and cell cycle kinetics. Since these processes are highly integrated, one could speculate that ethanol interference with caspase signaling is mediated by an inherent variability in the extent of programmed cell death that occurs naturally at different developmental stages. It is important to note that the present results are not exclusively related to CNS deficits, as the caspase activity measured here is whole embryo caspase activity and not confined specifically to the nervous system. With an axis of development, however, it is likely that a major portion of the measured caspase activity is related to the central or peripheral nervous system.

Defining critical windows for ethanol-induced brain injury is important for the rational design of novel neuroprotective strategies which may reduce the burden of abnormal neurodevelopment. This study demonstrates the responsiveness of medaka embryos to ethanol-induced inhibition of brain growth. Temporal and regional differences in this inhibitory effect were observed, indicating that the medaka CNS is highly sensitive to developmental ethanol toxicity. Brain volume was significantly decreased with ethanol exposure during early or late development, with embryos treated during mid-gestation remaining largely unaffected. This may reflect protective influences that effectively offset

CNS damage during this particular window of development. In addition, forebrain regions appeared more sensitive than hindbrain regions, though this treatment effect was not immediately apparent when viewing individual sections. Taken together, these findings indicate that the timing of gestational insult is a major determinant of developmental outcome in medaka embryos.

Table 4-1: Summary of Results for Chapter 4.

Endpoint	Window of Exposure		
	0-3 dpf	3-6 dpf	6-9 dpf
Caspase-3/7 Activity During Embryonic Development	↑14% on Day 1 ↑26% on Day 2	↓ 42% on Day 6	↓ 22% on Day 8
Caspase-3/7 Activity Immediately Before Hatching (Day 9 pf)	↓	↓	↓
Total Brain Volume	↓		↓
Rostral Brain Volume	↓		↓
Caudal Brain Volume	↓		

CHAPTER 5.
GENERAL DISCUSSION

Review of Accomplishments

This dissertation addressed the hypothesis that developmental ethanol exposure produces a phenotype in medaka that (1) varies depending upon the timing of exposure and (2) is qualitatively similar to human FAS. Ethanol-induced growth suppression is a common consequence of developmental ethanol exposure in mammalian systems and research described in this thesis shows that medaka embryos exhibit a similar response, demonstrating the sensitivity of the medaka early life stage assay for assessing developmental ethanol toxicity.

Morphometric data presented in Chapter 3 indicate dose-related decrements in brain and somatic growth which varied according to the precise timing of developmental ethanol exposure. Several parameters were initially chosen to assess ethanol effects on medaka growth and development, including measures of head width, head length, mouth length, neck width and total body length. Results from these investigations were consistent with human studies, which indicate that head width and total body length are the most sensitive growth parameters for assessments of developmental ethanol toxicity.

Approximately 60-70% of the nominal ethanol concentration was detected in treated embryos, suggesting that the intra-embryonic ethanol doses achieved in these

studies ranged from approximately 11.6 - 116 mM. Since it is unlikely that the outer chorion significantly restricts ethanol diffusion, these numbers should have been closer to 100%. In theory, other factors, such as embryonic metabolism or enhanced permeability due to acid washes may serve to limit ethanol analyses.

Despite extensive research on the adverse effects of developmental ethanol exposure, information regarding potential interactions between timing and toxicity is fairly limited. The medaka early life stage test was used to examine temporal variations in developmental ethanol toxicity and identify the critical period(s) for enhanced susceptibility. In order to investigate potential "windows" of heightened sensitivity, embryos were exposed to a range of ethanol concentrations during early, middle or late development and assessed for treatment-related effects on various measures of overt toxicity (*e.g.,* growth, hatching) and neurotoxicity (brain volume) (see Table 5-1 for a summary of the results). Hatching delays were found to be relatively insensitive to developmental ethanol exposure, exhibiting treatment effects only at the highest ethanol dose. Head width, a highly sensitive measure of ethanol toxicity, was decreased at all doses, regardless of the timing of exposure. Interestingly, the temporal pattern of ethanol sensitivity for treatment-related changes in brain volume and total body length appear to overlap, as decreases in both endpoints were observed when ethanol exposure occurred during the first or last developmental windows.

Assessments of caspase-3/7 activity were used to investigate treatment-related changes in apoptotic potential. These studies showed that ethanol exposure exerts differential effects on caspase-3/7 activity depending upon the window of exposure. Results obtained when caspase activity was measured sequentially throughout

development revealed increased caspase activation with early exposure (days 1 or 2 post fertilization) and decreased caspase activation with later ethanol exposure (days 6 or 8 post fertilization). When assessed on day 9 post-fertilization (after 72 hrs of ethanol exposure during early, middle or late development), however, comparable decreases in caspase activity were observed across all windows of exposure. Since there was no effect of window and a decrease in caspase activity was observed (instead of the expected increase), these results raise many questions regarding possible modes of action which require additional research.

Table 5-1: Summary of All Results by Window of Exposure

Endpoint	Window of Exposure		
	0-3 dpf	3-6 dpf	6-9 dpf
Gestational Length (Hatching)	↑ only at highest ethanol dose	↑ only at highest ethanol dose	No effect
Head Width	↓ at all doses	↓ at all doses	↓at all doses
Body Length	↓ at all doses	↓ only at highest ethanol dose	↓at all doses
Caspase 3/7 Activity During Embryonic Development	↑14% on Day 1 ↑26% on Day 2	↓ 42% on Day 6	↓ 22% on Day 8
Caspase-3/7 Activity Immediately Before Hatching (Day 9 pf)	↓	↓	↓
Total Brain Volume	↓		↓
Rostral Brain Volume	↓		↓
Caudal Brain Volume	↓		
Embryonic [Ethanol]	60% of nominal	66% of nominal	69% of nominal

Data in the shaded region represent exposure to only 1% ethanol.

General Discussion

"A bewildering number of possibilities..." this was the consensus regarding potential mechanisms for developmental ethanol toxicity two decades ago and it remains largely unchanged today. A wide range of hypotheses have been proposed for the teratogenic effects of developmental ethanol exposure, but as yet, no primary mechanism has been identified with any certainty. In many ways, the field is still wide open, as researchers continue to identify new targets within the complex spectrum of effects associated with prenatal ethanol exposure. The current state of evidence regarding developmental ethanol toxicity seems to indicate multiple effects on multiple systems with no real indication of whether early or late ethanol exposure is more hazardous to the developing embryo (Table 5-1).

Results obtained in the present study lend support to this notion. I have used medaka embryos to investigate temporal variations in developmental ethanol toxicity and my findings suggest that there is no finite period in development that is most or least sensitive to ethanol. Given the complexity and precision of events required for normal development, a logical assumption might be that the critical interval for developmental ethanol exposure is widespread, manifesting various degrees of toxicity depending upon the precise timing of exposure.

The ethanol dose is known to be a critical determinant of developmental outcome, so it is unfortunate that relatively few investigators have attempted to calculate the intra-embryonic ethanol concentration following developmental exposures. This shortcoming limits data interpretation, as it is very difficult to make meaningful comparisons between studies. I have conducted a series of experiments that investigate the dose-response

relationship for developmental ethanol toxicity in medaka embryos. Using an optimized kinetic assay for ADH-mediated NADH accumulation, it was determined that medaka embryos take up approximately 60-70% of the waterborne ethanol concentration. This value is higher than previous estimates in zebrafish (~30%) and medaka embryos (20%) (Reimers *et al.* 2004; Wang *et al.* 2006a). Although I cannot offer a definitive explanation for this disparity, two methodological factors are of particular note; a longer exposure interval and saturating amounts of the cofactor NAD were used in the present ethanol assessments. It is possible that these modifications to the original protocol may have altered the extent of ethanol accumulation in the embryo or the reaction kinetics for NADH accumulation. Our findings, however, are compatible with an earlier study in goldfish which reported that ethanol rapidly achieves steady state in brain with tissue concentrations that are comparable to the waterborne ethanol concentration (Greizerstein and Smith 1973).

The intra-embryonic ethanol dose attained in our experiments ranged from 12.0-120 mM (0.07-0.7 %) after three consecutive days of ethanol exposure (0.1-1.0%). Although the highest ethanol dose used in these studies may be considered irrelevant to humans, it is comparable to that seen in other animal models of FAS. For example, rabbit embryos exposed to 154 mM ethanol from GD 9-11 were shown to develop cardiac and craniofacial abnormalities (Pitt and Carney 1999), whereas similar exposures in rodents (60 – 152 mM) from GD 6-16 produced craniofacial and neural tube defects (Becker *et al.* 1996). In another, more extreme exposure paradigm, zebrafish embryos exposed to 2.4% ethanol (~ 425 mM) developed cyclopia which was accompanied by a loss of gene expression (sonic hedgehog; homebox nk2.2) in ventral forebrain and midbrain regions

(Blader and Strähle 1998). Because the ethanol concentrations used in our experiments are within range of those used in previous studies, they are relevant for comparisons with other animal models of FAS.

There have been relatively few studies of ethanol-induced teratogenesis in developing fish (Carvan *et al.* 2004; Reimers *et al.* 2004; Wang *et al.* 2006a), but the literature suggests that ethanol affects fish development in a manner which is similar to effects seen in mammals and birds (Abel and Dintcheff 1978; Baumann and Sander 1984; Cartwright and Smith 1995; Ghishan *et al.* 1982). A number of studies have documented ethanol-induced increases in stress proteins (Krone *et al.* 1997; Lele *et al.* 1997), spinal cord malformations (Laale 1971) and flexures of the body axis (Baumann and Sander 1984) in zebrafish embryos. Our results indicate that medaka embryos are also highly sensitive to developmental ethanol exposure. Initial range finding experiments revealed that embryonic ethanol exposure at or above 2% is lethal during medaka development. In addition, exposures below this concentration produced dose-related deficits in medaka growth and development. Endpoints scored in these studies include hatching success and various measures of somatic growth. Of these, total body length and head width appeared to be most sensitive to developmental ethanol exposure. Embryos were relatively insensitive to ethanol-induced hatching delays, exhibiting treatment effects only at the highest dose. This high dose phenomenon may reflect ethanol-induced inhibition of the generation and/or migration of hatching gland cells. The fact that lower ethanol doses were ineffective suggests that the length of gestation may be "hard-wired"in the embryo, requiring intense disruptions that are overtly toxic in order to induce changes.

The timing of developmental ethanol exposure is known to be a critical determinant of toxicity. Windows of heighted sensitivity to developmental ethanol toxicity have been identified in a number of species (Cartwright and Smith 1995; Coles *et al.* 1991; Tran *et al.* 2000; West *et al.* 1986). The pattern of effects described in Chapters 3 and 4 indicate that temporal variations in ethanol toxicity also exist for medaka embryonic development. When exposed during early development (0-3 dpf), medaka embryos were found to be extremely sensitive to ethanol toxicity, exhibiting treatment related decreases in head width and total body length. A similar trend was observed with ethanol exposure late in development (6-9 dpf), indicating that this developmental window is also highly sensitive to ethanol exposure. It is intriguing to note however, that when ethanol was administered during the middle portion of development, embryos were less responsive to ethanol-induced decreases in total body length and brain volume. This may reflect the presence (or absence) of a specific cellular constituent at this particular time interval which effectively modulates toxicity. It could also indicate overlap between critical events that occur during early or late development. The lack of a precise temporal delineation of beginning or end, due to asynchronous development between embryos could skew results. In this case, responses to ethanol exposure would be expected to vary considerably during the interim between sensitive developmental events that occur early or late in gestation, which could theoretically mask treatment effects.

No single mechanism can account for all of the adverse effects that have been observed in FAS, but animal models have identified several possibilities, many of which culminate as cell death via apoptosis or necrosis. There is also considerable evidence for ethanol-induced caspase-3 activation in the literature (Carloni *et al.* 2004; Climent *et al.*

2002; Light *et al.* 2002; Mooney and Miller 2001; Ramachandran *et al.* 2001). Though often viewed in terms of apoptotic potential, the relationship between caspase-3 and cell death is not absolute. Modulations in caspase activity may also induce subtle changes in the orderly sequence of events that orchestrate normal embryonic development. Since the total cell number of an embryo is determined via summation of additive (cell proliferation, neuronal migration and neurite outgrowth) and subtractive (pruning and neuronal cell death) processes and ethanol affects all of these developmental events (Bhave and Hoffman 1997; Borges and Lewis 1983; Climent *et al.* 2002; Luo and Miller 1997; Pantazis *et al.* 1993; Vaudry *et al.* 2002; Zhang *et al.* 1998a), it is unlikely that a change in caspase activation would be the sole determinant of ethanol-induced decreases in cell density.

Although developmental ethanol exposure has been shown to decrease neuronal survival, recent evidence suggests that all neurons exhibiting the common cell death markers (*e.g.*, TUNEL, caspase) do not die (Cheng and Zochodne 2003; Gordon *et al.* 2002; Ishida *et al.* 2004; Oomman *et al.* 2004). In fact, stereological estimates indicate that the loss of cortical neurons following developmental ethanol exposure is less than 33% (Mooney *et al.* 1996; Mooney and Napper 2005). Perhaps the switch for caspase activation is incremental rather than ON/OFF. In some instances when appropriate neuronal cues are not present, the damage which triggers caspase activation may actually represent a "sublethal" insult that causes developing circuits to enter a "waiting stage" in order to allow time for the necessary input (or more favorable conditions) to become available.

In the present study, all animals exposed to 1% ethanol during development exhibited substantial, treatment-related changes in caspase-3/7 activity, however the nature of the observed changes varied with the timing of exposure. Early ethanol exposure increased, whereas mid to late gestational exposure decreased caspase-3/7 activity. A similar trend has been observed in chick embryos. In this model, the extent of ethanol-induced cell death was increased relative to controls with early ethanol exposures (gastrulation or neurulation), but not with those that occurred later in development (*e.g.*, 16 somite stage) (Cartwright and Smith 1995).

Though it is generally well-accepted that increased caspase-3/7 activation can be equated with increased apoptosis, it is becoming increasingly clear that this long held notion may be too simplistic, as caspase-3/7 is known to possess non-apoptotic functions as well. For example, caspase-3/7 has been shown to play a pivotal role in cellular differentiation [(Ishizaki *et al.* 1998); for recent reviews see Abraham and Shaham 2004; Launay *et al.* 2005; Sehra and Dent 2006] and neuronal pruning (Kozlowski *et al.* 1997; Miller 1987). Since these developmental processes are highly integrated, it is appealing to speculate that the observed spatiotemporal variations in ethanol-induced caspase activation may, to some degree, reflect normal fluctuations in the extent of programmed cell death that occurs naturally during different stages of development. It is important to note however, that the present results reflect whole embryo caspase activity and as such, are not exclusively related to nervous system development.

The relationship between caspase activation and programmed cell death can be ambiguous. Our results are consistent with a recent study in rats that reported ethanol-induced decreases in caspase-3 activity in the pancreas (Wang *et al.* 2006b). Though

somewhat counterintuitive, the idea that ethanol might decrease caspase activity is not inconceivable. In some situations where caspases are normally activated, their inhibition simply delays, rather than prevents cell death (D'Mello *et al.* 2000; Keramaris *et al.* 2000; Selznick *et al.* 2000; Stefanis *et al.* 1999). Hence changes in caspase activation may not directly correlate with spatiotemporal patterns of cell death.

Developmental ethanol exposure is known to decrease cortical expression of pro-survival proteins (Mooney and Miller 2001), whereas transgenic over-expression of Bcl-2 has been shown to protect cerebellar Purkinje neurons from ethanol-induced cell death (Heaton *et al.* 1999). In addition, net decreases in the Bcl-2/Bax ratio observed in rat brain were shown to coincide with periods of enhanced ethanol-induced cell death in cortical proliferative zones (Mooney and Miller 2001), suggesting a critical role for these proteins in apoptotic signaling. A lack of ethanol-related changes in Bcl-2 or Bax expression was observed in the ventrolateral nucleus of the thalamus, a structure that appears relatively insensitive to ethanol-induced cell death (Livy *et al.* 2001). An ethanol effect on cell death factors related to p53 signaling has also been surmised. The temporal pattern associated with ethanol-induced changes in p53 expression resembles that seen with ethanol-induced changes in Bcl-2 expression; ethanol exposure during gestation increased prenatal p53 expression in rat cortex (Kuhn and Miller 1998). Chronic prenatal ethanol exposure was also shown to increase p53 expression in rat cerebellum during the early postnatal period (de la Monte and Wands 2002). Implicit in these findings is the notion that the functional reserve for CNS recovery processes may vary significantly across brain regions and stages of development. That is, observed variations in the

inherent sensitivity to ethanol-induced cell death may reflect region- and time-specific gradients in the relative abundance of pro-survival and/or apoptotic effector molecules.

Morphometric studies in rodents treated with ethanol during development have shown that the size of the brain may be as much as 12-30% smaller in treated animals relative to controls (Pentney and Miller 1992). Variability in the sensitivity to this response may reflect differences in the method of ethanol administration and/or the timing of exposure (Lancaster *et al.* 1982; Miller and Potempa 1990; Pierce and West 1987; Randall *et al.* 1977; Samson and Diaz 1981; Stoltenburg-Didinger and Spohr 1983). I have addressed temporal variations in ethanol toxicity, using a consistent intra-embryonic ethanol dose for all comparisons across early, middle or late development. Since there is a strong correlation between developmental ethanol-exposure and growth retardation, this growth inhibitory phenomenon was used to determine which interval of medaka development is most susceptible to ethanol toxicity. Results obtained from initial morphometric studies implicate the first and last developmental windows as particularly sensitive periods for ethanol-induced growth inhibition. However subsequent assessments of ethanol-induced changes in brain volume clearly indicate that the first developmental window is most sensitive to ethanol-induced inhibition of brain growth. Since Adh8 expression (which is considered to be functionally analogous to human ADH1) is not increased until ~72 hpf (Dasmahapatra *et al.* 2005) this may partially explain the increased growth inhibition observed in embryos treated during the first exposure window. The temporal relationship between changes in embryonic capacity for ethanol metabolism and heightened sensitivity to developmental ethanol exposure is unclear. Once absorbed, ethanol distributes rapidly within the total body water and readily diffuses

across cell membranes (reviewed in Rogers and Daston 1997), where it is oxidized to acetaldehyde by alcohol dehydrogenase, CYP2E1 and catalase. Of these, ADH1 is responsible for the bulk of ethanol metabolism in the liver (Boleda *et al.* 1989).

Although direct (ethanol) and indirect (acetaldehyde) mechanisms have been proposed for the negative effects of developmental ethanol exposure, a recent zebrafish study showed that inhibition of ethanol metabolism dramatically increased the incidence and severity of ethanol toxicity in developing zebrafish embryos (Reimers *et al.* 2004). A number of other studies support the contention that metabolic conversion of ethanol to acetaldehyde is not necessary for inducing teratogenic effects *in vivo*. For example, dose-dependent increases in lethality and malformations were enhanced in rats and mice that were co-exposed to ethanol and 4-methyl pyrazole, an inhibitor of alcohol dehydrogenase (Blakley and Scott 1984; Chen *et al.* 1995). This notion is further strengthened by the fact that ethanol sensitivity was shown to be inversely related to ADH activity in the fruit fly (*Drosophila melanogaster*). In these studies, flies with the lowest ADH activity had twice as many malformations as those that possessed high ADH activity (Ranganathan *et al.* 1987). These findings are consistent with morphometric data presented in this thesis which indicate that medaka embryos are most sensitive to ethanol-induced growth inhibition during the first 72 hours of development, when Adh8 expression is minimal.

Another possible explanation relates to temporal variations in the cellular environment which may impact adaptive responses to ethanol toxicity. For example, neurotrophin expression and neuronal dependence on trophic support vary in a region- and time-dependent manner during development. Purkinje cells switch from BDNF to NT-3 support between P4 and P6 (Davies 1997), which is a period of heightened

sensitivity to ethanol exposure (Light *et al.* 2002). Developmental ethanol exposure may interrupt neurotrophic signaling in a number of ways, depending upon the neuronal population and the time of exposure. Pre- (Miller and Potempa 1990) and postnatal (Mooney *et al.* 1996) ethanol exposure have been shown to produce comparable decreases in rat cortical neurons. Interestingly, treatment-related decreases in neuronal density were coincident with changes in gene expression profiles that influence neuronal death. For example, Bcl-2 and caspase-3 expression normally increase in the neocortex during the first two weeks of postnatal development, but ethanol exposure effectively delayed this up-regulation (Mooney and Miller 2001). We observed similar treatment-related delays in caspase-3 activation following ethanol exposure in medaka embryos.

Although ethanol-induced growth inhibition is a salient feature of developmental ethanol toxicity, a more comprehensive strategy may prove useful for identifying critical windows for developmental ethanol toxicity. It is possible that periods of heightened sensitivity are not adequately defined by chronological age. A more accurate representation of these developmental intervals may require incorporation of elements associated with the immediate cellular environment. The mere fact that there are so many different manifestations of developmental ethanol toxicity suggests that ethanol-induced physiological responses may be tempered by the "tone" of the microenvironment in an affected region at the time of exposure. This phenomenon has been documented in the ethanol literature, where, in addition to its ability to destroy cerebellar (Li *et al.* 2002) and cortical (Jacobs and Miller 2001) neurons, developmental ethanol exposure has been shown to delay proliferation of cerebellar (Li *et al.* 2002) and cortical (Miller *et al.* 2003) progenitor cells and to decrease the rate of neuronal migration (Miller 1993). These

compensatory responses are not necessarily maladaptive. They most likely represent an attempt to re-establish homeostasis and in so doing, serve to promote survival. Embryonic development is a dynamic process which is highly responsive to a myriad of intracellular and environmental cues. As such, the delineation of critical periods for enhanced sensitivity may not be one dimensional.

Future Directions

A growing body of evidence indicates that genetic background can modulate the addictive and neurobehavioral properties of ethanol. Studies in rodents, birds, and humans indicate that sensitivity and resistance to ethanol toxicity is influenced by the genotype of the organism (Bruyere and Stith 1993; Bupp Becker and Shibley; Fatayerji *et al.* 1996; Loucks and Carvan 2004; Pecsenye *et al.* 2004). Genetic polymorphisms in ethanol metabolizing enzymes (Chernoff 1980; Itoga *et al.* 1999; Whitfield *et al.* 1997), neurotransmitter systems (Gelernter *et al.* 1997; Parsian *et al.* 1996) and quantitative trait loci (Crabbe *et al.* 1999) have been identified. Other modulators of developmental ethanol toxicity may be independent of maternal metabolism, as demonstrated in crosses between susceptible (Long Sleep) C57BL/6J and resistant (Short-Sleep) C57BL/6J mice. In these studies, Long Sleep, but not Short Sleep mice experienced growth (Goodlett *et al.* 1989) and learning (Gilliam *et al.*) deficits. The medaka early life stage assay is a relatively simple developmental model that has the potential to advance our understanding of differential susceptibility to ethanol-induced teratogenesis. Genetically distinct medaka strains could be used to address this possibility, by comparing gene expression profiles for ethanol exposed and control embryos in order to identify candidate genes that may

contribute to differential sensitivity. Utilizing cDNA microarray techniques, researchers can test numerous end points simultaneously. Those strains which exhibit heightened sensitivity could later be subjected to learning and memory (or drug challenge) studies to determine the longterm consequences of developmental ethanol exposure. The ability to correlate changes in gene expression with changes in behavior and cognitive function will facilitate rapid progress in this research area.

An efficient and cost-effective means to ascertain risk is perhaps the most widely sought commodity in hazard management. Research presented in this thesis demonstrates the sensitivity and reliability of the medaka early life stage assay as an alternative approach for assessments of ethanol and possibly other, known or suspected developmental toxicants.

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