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Behavioral Changes in Adult C57BL/6J Mice following Prenatal Exposure to Ethanol.

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by

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ABSTRACT

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Fetal Alcohol Syndrome (FAS) labels children with physical, mental and behavioral deficits exposed to alcohol *in utero*. Current research indicates that timing of alcohol exposure of the embryo/fetus is a critical determinant of the behavioral deficits associated with FAS. This study represents a model for binge drinking, in which C57BL mouse embryos were exposed to alcohol during 2 separate critical periods of brain development, gestational day (GD) 7 or 8. As adults, the offspring were tested to determine if loco-motor activity and emotional reaction to a novel environment had been affected. Significant differences due to treatment and sex were noted for both the number of urinations ($p=.005$ and .001, respectively) and fecal boli ($p=.011$ and .001, respectively). These results suggest that the quantity of alcohol exposure *in utero* on the developing brain as in this binge-drinking model is critical in terms of adverse effects on behavioral outcome for the offspring.

DEDICATION

This work is dedicated to my mother and father who always support my dreams no matter what they may be and to my friends for giving me love, laughter, and excitement over the years.

“A sailor who has no destination knows neither a good wind from a bad wind”

-author unknown

Thank you all for being there for me when I needed you.

AKNOWLEDGEMENTS

In appreciation of my committee: Dr. Michael Woodruff, Dr. Ron Baisden, Dr. Darryl Moore, and especially Dr. Uta Schambra for all of their time and effort in helping and supporting me along this long road. Without them, I would not have made it to this point.

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CHAPTER 1

INTRODUCTION

There are a variety of non-preventable birth defects or disorders that can occur while the fetus develops. Some specific examples include Down Syndrome, Cerebral Palsy, and San Filippo. However, the birth defect that is the leading cause of mental retardation in both North America and Europe, Fetal Alcohol Syndrome, is 100 percent preventable. In France, Sweden, and North America, 1 out of every 750 live births per year is diagnosed with Fetal Alcohol Syndrome. Although a description of Fetal Alcohol Syndrome was first published in 1973 in the U.S., alcohol use among pregnant women has increased 4-fold from 1991-1998 (Caleekal 1999). Alcohol enters the infant's blood stream through the mother's placenta and fetal alcohol concentration can equal maternal blood alcohol concentration. Because alcohol is soluble in both water and fat, it easily permeates all tissues in the fetus, causing interference with cell development in the embryo.

Dr. Paul Lemoine and his colleagues in France first observed Fetal Alcohol Syndrome in 1968. They began to notice that children of known alcoholic mothers were developing birth defects that followed a definite pattern. In 1973, two University of Washington researchers, David Smith and Kenneth Jones, also described a specific pattern of birth defects observed in children with alcoholic mothers and coined the term Fetal Alcohol Syndrome (Lee 2001). Since the discovery and subsequent labeling of FAS, more than 2000 studies

have been conducted and have confirmed that alcohol consumption while pregnant can cause permanent birth defects in the offspring.

In a broad sense, Fetal Alcohol Syndrome could be viewed as a direct relation of an external environmental influence on the internal physiology of the developing fetus (Caleekal 1999). Clinical diagnosis of alcohol related disorders occur in 1of 2 categories: Fetal Alcohol Syndrome (FAS) and Fetal Alcohol Effects (FAE).

Diagnosis of FAS is based on 3 criteria: a growth deficiency either prenatally or postnatally in height or weight, a specific pattern of facial and physical abnormalities, and a central nervous system dysfunction (Randels and Streissguth 1992). The growth deficiencies may result in several different structures being affected and singular or multiple growth disorders can occur. The body weight and/or size may be smaller than normal. The skeleton may be affected by alcohol in adverse ways. The ribs and sternum may be deformed, the spine may be curved, the hips may be dislocated, fingers and toes may be bent irregularly, fused, webbed, and/or missing, joints may be stiffer and have more limited movements and the cranium may be smaller than average.

Facial abnormalities in FAS may include smaller eyes with smaller openings and/or a webbing of skin between the eyes and the base of the nose. Additionally, eyelids may droop, nearsightedness may develop, and the eyes may be unable to move in the same direction. The child may have a sunken nasal bridge, upturned small nose, and a flattened or absent groove between the upper lip and nose, an opening may be present in the roof of the mouth, the jaw may be smaller than average, and the ears may be poorly formed. Figure 1

indicates several of the facial deformities listed previously. Internal systems may also be compromised and different organs may be affected. The heart may develop murmurs or defects, the genitals may be malformed, and the kidneys or urinary tract may be damaged.

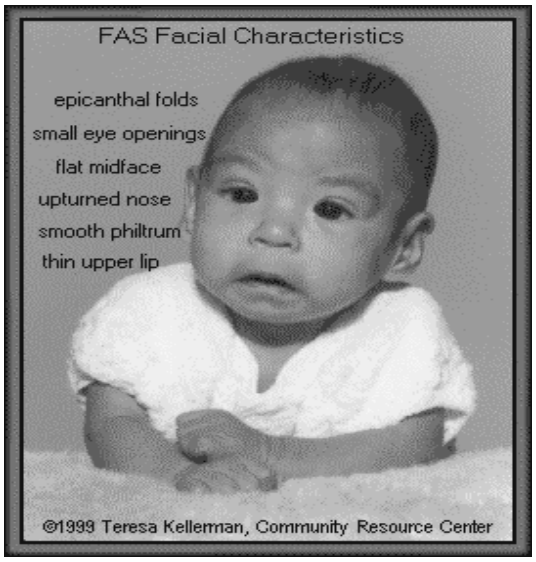

Figure I. Characteristic Pattern of Facial Features

The last and possibly most detrimental effect of alcohol on a fetus involves the central nervous system. The effects that occur in the central nervous system may involve structural defects, neurological dysfunction, or behavioral problems. Structurally, microcephalism and small brain may occur, faulty arrangement of brain cells, or damage to specific structures of the brain. Neurologically, seizures, abnormal muscle tone, tremors or uncoordination, as well as hearing and visual problems may occur. At the behavioral level of analysis, attention deficits, impaired reasoning, mild to severe mental retardation, impaired judgment, speech delays, language delays, learning disabilities, short attention span, irritability in infancy, and hyperactivity have been observed (Abel 1986).

If the child only has 1 or 2 of the symptoms listed above and a clear history of maternal drinking is present, then the child is said to have Fetal Alcohol Effects (FAE). A diagnosis of FAE is more frequent than diagnosis of full-blown FAS. FAE is estimated to occur 3 – 10 times more often than FAS (Smith 1987). An example of a child with FAE versus FAS could be a child with both growth deficiencies and CNS impairment but no facial or cranial abnormalities.

There is no eradication of the effects of alcohol on the fetus. Once the damage has been done *in utero*, the child will have some level of impairment. As the child progresses and ages, he or she will continue to have social and cognitive problems. As adolescents and adults, the child may display poor social judgment, and may have more difficulty with reading, arithmetic, and spelling and general intellectual functioning and adaptive skills tend to remain below average. It is important to note that there can be great variability in individuals due to environmental and psychological supports that may be more readily available to some than others (Burgess and Streissguth 1990).

From the time FAS was discovered it has been the source of fuel for many research studies. For the most part, researchers have concentrated on the chronic administration of alcohol to experimental animals i.e. ethanol treatment of a pregnant dam during all of gestation, and have evaluated the neurobehavioral and physical effects that may occur as a consequence in the offspring. In several studies, rats exposed to alcohol have shown delays in growth, developed hyperactivity, and shown deficits in learning and memory (Friedler 1988). However, these studies relate to the birth defect in children of chronic alcohol abusers and not binge-drinking mothers. In binge drinking models using C57BL

mice, it has been shown that ethanol exposure on only one day representing a critical period of embryonic development can lead to specific damage of brain structures and other organ systems. For instance, Randall and Taylor found that ethanol exposure on any day from the 5th through 10th gestationally resulted in neurological deficits structurally and that the incidence of fetal wastage and birth defects increased (Randall and Taylor 1979). Another study found that acute maternal administration of ethanol in mice on gestational day (GD) 7 has been shown to result in a variety of eye malformations but these same malformations did not occur on GD 8 indicating a specific period of developmental disruption due to ethanol exposure (Cook et al. 1987). Thus, it is important to understand the differences in timing of alcohol exposure during certain periods of development in terms of severity of defect and different systems being affected.

During the first 8 weeks of human pregnancy, the developing nervous system is most vulnerable. In addition to the central nervous system, during this time frame the eyes, legs, arms, teeth, head, palate, and external genitalia also develop and may be affected by alcohol exposure. Thus, these malformations can serve as indicators of developmental disruption at a specific time or critical period for a particular structure (Caleekal 1999). We chose to investigate the behavioral effects of binge-drinking during 2 critical periods of brain development that lie within this time frame, based on earlier studies that histologically show damage to different brain structures.

One of the earliest critical periods of brain development that resulted in neuronal deficit after exposure to ethanol was gestational day 7. In a study by Sulik, Johnston, and Webb, C57BL pregnant mice were exposed to alcohol on

GD 7. The resulting embryos had decreased development in the neural plate and craniofacial abnormalities that resembled those seen in human FAS (Sulik et al. 1981). This was later elaborated on by Sulik and Johnston who found that 24 hours after exposure to ethanol on GD 7, C57BL/6J mice fetuses held marked skeletal deficiencies, size reduction in the forebrain region, and again facial abnormalities (Sulik and Johnston 1983). Other research indicates that ethanol exposure on GD 7 affects the forebrain and that the neurotransmitters affected by the insult of ethanol could be an explanation for the functional deficits that are observed in offspring of alcoholic mothers (Schambra et al. 1990). These studies represented a mouse model to a maternal drinking binge on a Friday and Saturday night during the third week of pregnancy. In this model, exposure of pregnant mice to 2 doses of ethanol on gestational day 7 resulted in severe defects of forebrain structures and the eyes (Sulik et al. 1981, Sulik and Johnston 1983, Schambra et al. 1990).

The effects of ethanol exposure on a mouse embryo on GD 8, equivalent to the 4th week of human gestation, however, resulted in a different set of defects. During this critical period, cells at the posterior neural plate were affected and resulted in changes in the hindbrain structures. This led to malformations such as exencephaly, pituitary dysplasia, and cleft palates while the eye abnormalities previously seen on GD 7 were no longer noted (Cook et al. 1987, Kotch and Sulik 1992). Based on the histological differences found by other researchers between GD 7 and GD 8, we performed this study, fully expecting similar differences in behavioral outcome to occur.

At East Tennessee State University, Dr. Schambra and others have begun looking more closely at the effects of both timing of exposure to alcohol during pregnancy and quantity of alcohol given to the mother on structural brain development and subsequent behavior. The hypotheses to be tested in this project are (1) that exposure to alcohol during 2 different gestational stages will affect different parts of the brain resulting in different behavioral changes in the adult off-spring and (2) that there will be a dose-response relationship with higher doses of alcohol causing more severe disruption of development and more profound behavioral changes in the offspring.

CHAPTER 2

EXPERIMENTAL DESIGN

Mating

C57BL/6J mice were obtained from the Charles River facility in Raleigh, NC. The mice were housed in the AAALAC accredited colony facility at East Tennessee State University with commercial lab chow and water available *ad libitum*. A 12/12 light cycle was maintained in the colony room with lights off at 8:00 am. A single, sexually mature (70 days or older) male was placed with a single, sexually mature female everyday for a 1hour period of time beginning at 8:00 am. The time increment was kept at one hour to assure consistency for gestational timing. Successful copulation was determined by the presence of a vaginal plug and that day was considered as gestational day 0, 0 hours. The female was given an identification number, weighed, and assigned to either 1 of the 4 control groups or to 1 of the 4 treatment groups. On GD 7, the mouse was reweighed and pregnancy verified if she had gained at least 1 gram.

Treatment

The groups consisted of pregnant dams to be treated and their offspring to be tested. To assess whether neonatal testing (part of a separate project) or gastric intubation had an effect on the behavior of the offspring, we divided the animals into 4 control groups. These groups consisted of: (1) Untreated Controls (UC); (2) Gavage Controls treated on GD 7 (GC-7); (3) Gavage Controls treated on GD 8 (GC-8); and an Untreated Control groups whose pups were the only pups Not Neonatally tested (UCN). Because all other pups were involved in a

neonatal test battery evaluating their sensori-motor development (not part of this thesis), we were concerned that the extensive handling of these pups might affect later behavioral outcome. The UCN group was formed to identify any changes.

The choice of ethanol dose was based on the literature which had documented that 2 doses of 2.9g/kg of maternal body weight, given as a 25% solution, was the highest dose tolerated with minimal fetal wastage (Sulik et al. 1981). Our preliminary studies showed that this dose given by gastric intubation produced a blood alcohol concentration (BAC) of 200mg% in our mice shortly after the second dose was administered. We arbitrarily set the lower dose at 2 doses of 2.4 g/kg, which produced a BAC of about 150mg%. These 2 treatment groups were then used on either GD 7 or GD 8. Ethanol treatment by gastric intubation was performed on GD 7 at O and 4 hours, i.e. at 8 am and noon, and on GD 8 at 6 and 10 hours, i.e. at 2 pm and 6 pm. The 4 resulting treatment groups were labeled by amount of ethanol and given in 1 of the 2 doses and on by gestational day, thus forming these groups: (1) 2.4 -7; (2) 2.9-7; (3) 2.4 -8; and (4) 2.9 - 8.

For the behavioral assessments, all 4 control and 4 treatment groups were further subdivided into male and female offspring, thus originally resulting in a total of 16 groups.

Testing

All newborn pups, except group UCN, underwent neonatal testing beginning at postnatal day 3. Neonatal testing consisted of negative geotaxis, horizontal screen, crawling/spinning, righting reflex, cliff aversion, and open-field

tests. When the mice reached postnatal days 65, all mice were tested for 3 consecutive days in an open field box to determine if locomotor activity and emotional reaction to a novel environment had been affected by their prenatal exposure to alcohol.

The open field box measured 1 meter on each side and was divided into 36 equal area squares by painted lines on its floor. The mouse to be tested was placed into the center of the box and its behavior observed for 10 minutes indirectly with a video camera under normal room illumination from a separate room to exclude distractions The number of lines crossed and the number of times the mouse reared onto its hind legs in the 10-minute interval served as the measure for activity and the number of fecal boluses and urinations were also recorded as a measure of emotional response.

CHAPTER 3

RESULTS

The Bartletts' Test for homogeneity of variance was applied to the data for each dependant variable. Where the results of the Bartletts' Test indicated that the data did not fit the assumption of homogeneity of variance that underlies the use of parametric statistical tests, non-parametric analyses for statistical significance were used.

For each dependant variable the untreated control group (UC) was compared to each gavage control group (GC-7 & GC-8) using Analysis of Variance. The control groups did not differ from one another on any of the dependant variables. For activity, the comparison of UC versus GC-7 and GC- 8 yielded $F(1,13)=1.31$, $p=.275$ and $F(1,13)=1.08$, $p=.317$ respectively. For rearing, $F(1,13)=.03$, $p=.874$ and $F(1,13)=.05$, $p=.830$. For bolus, $F(1,12)=.01$, $p=.905$ and $F(1,13)=.12$, $p=.739$. In urine, $F(1,12)=.19$, $p=.608$ and $F(1,13)=.13$, $p=.72$. Therefore, to increase statistical power, the control groups were combined. Because there were 2 gavage control groups, 1 for each exposure day (GC-7 and GC-8), the UC group was divided in half. The data from one half of the UC mice were combined with the data from mice in the GC-7 group. The data from the other half of the UC mice was combined with the data from the GC-8 group. These combinations formed new combined control groups CC7 and CC8, respectively. Analyses of the results are presented in a separate section for each dependant variable: open field activity, rearing, fecal boli, and number of urinations.

Behavioral Analyses

Open Field Activity

The Bartletts' Test applied to the activity variable as assessed by the total number of lines crossed during the three testing sessions yielded a significant p-value (See Figure 2.) For that reason the Kruskall-Wallis nonparametric analysis of variance was applied to the data.

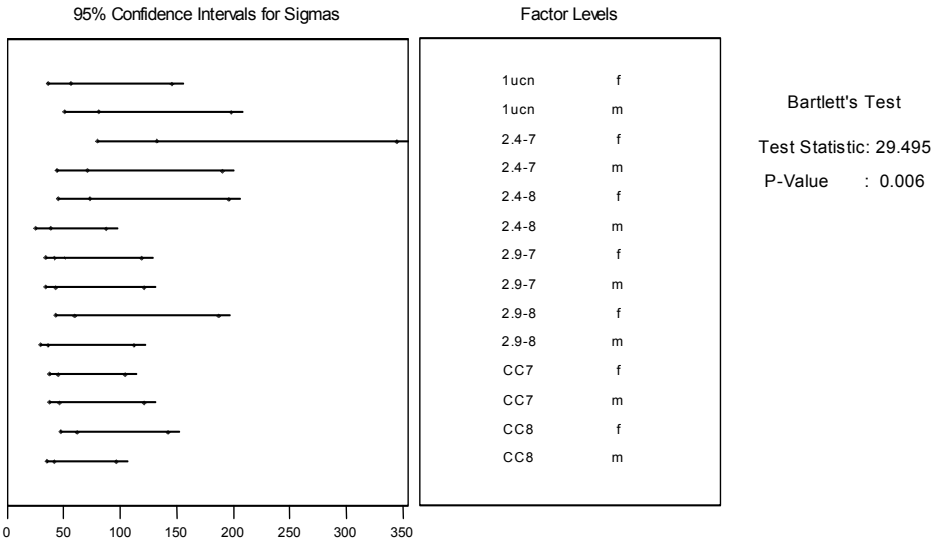

Figure 2. Test for Equal Variances for Activity

Because the test employed to statistically access the number of lines crossed is non-parametric, the median for each group is presented in Figure 3 to represent the activity for each group.

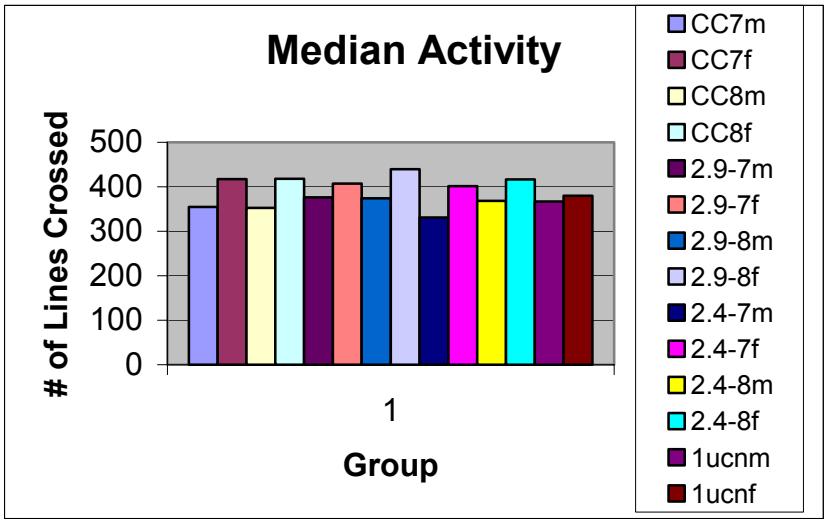

Figure 3. Median Histogram for Activity per Group

The Kruskall-Wallis non-parametric test was applied to the total number of lines crossed resulting in the treatment effect not being significant H=4.89, DF=6, p=.558. However, the effect of sex on activity level was significant DF=1, p=.001.

Rearing

A p-value of .097 indicates that the level of variance between groups for rearing is closer to equal and therefore more homogenous (Figure 4).

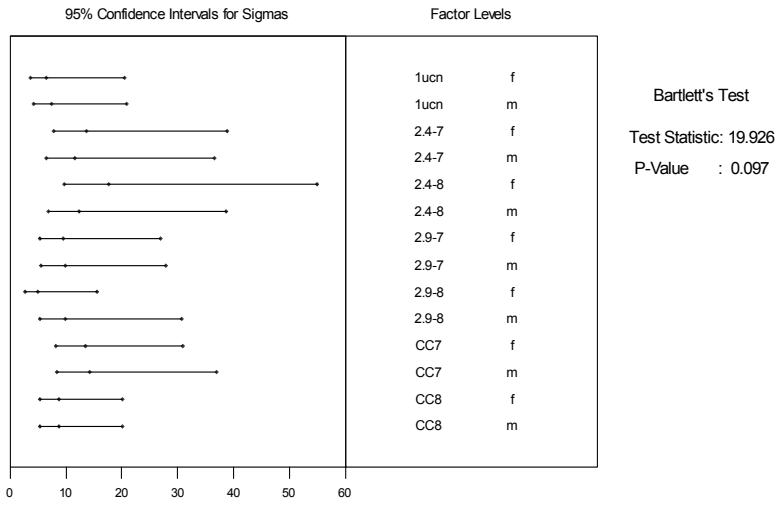

Figure 4. Test for Equal Variances for Rearing

Another visual depiction of variance is a plot of the medians in a histogram for each group (Figure 5).

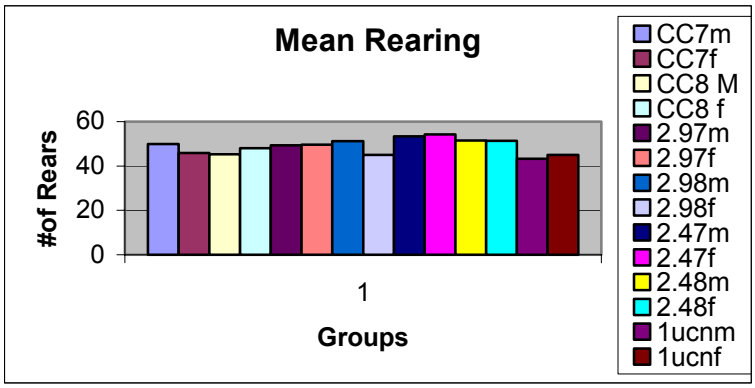

Figure 5. Mean Level of Rearing Per Group

Application of a two-factor (treatment by sex) parametric analysis of variance to the total number of rears did not yield either a significant treatment effect F(6,116)=1.43, p=.210 or a statistically significant sex effect F(1,116)=.73, 9=.716.

Bolus

The Bartletts' test indicated a significant lack of homogeneity of variance for bolus output between the groups with a p-value of .000 (Figure 6).

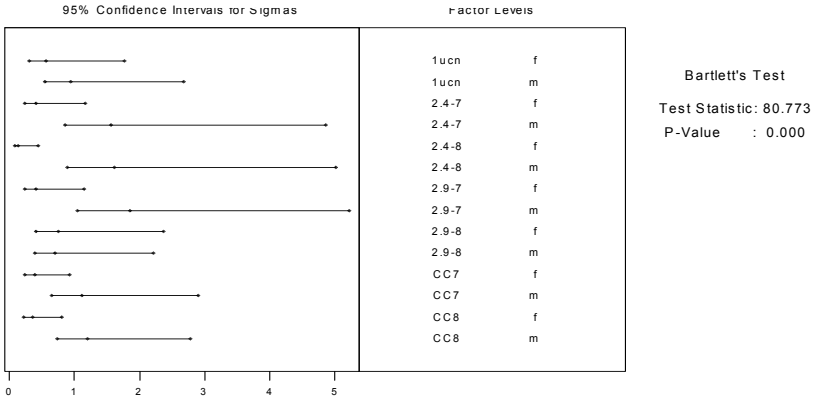

Figure 6. Test for Equal Variances For Bolus Output

The median histogram for bolus also gives a strong visual depiction of the differences among the groups in relation to bolus output (Figure 7).

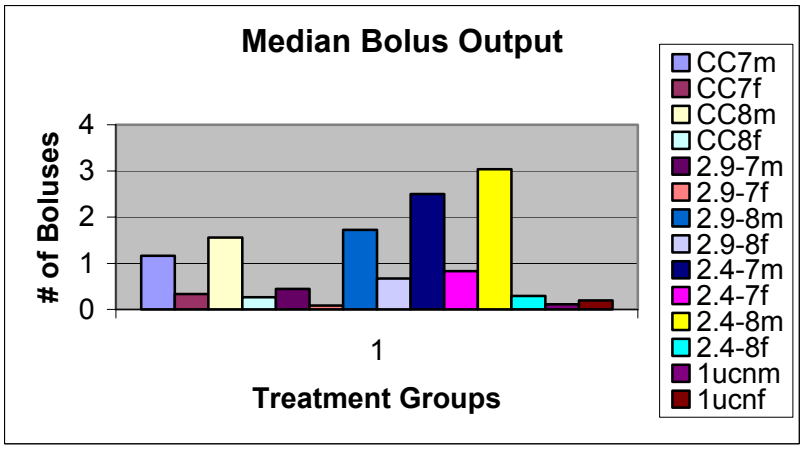

Figure 7.Median Histogram for Bolus Output per Group

When the Kruskall-Wallis test was applied to the amount of fecal boluses produced, there was a significant treatment effect H=15.41, F=6, p=.017 and a

significant sex effect H=28.28, F=6, p=.001. To determine where the differences were between groups, Mann-Whitney tests were performed. Significant differences were found between the following groups:

CC7m vs. CC7f, U=151.0, p=.0182,

CC7m vs. 2.4-7m, U=70.0, p=.0291,

CC8m vs. CC8f, U=190.0, p=.0219,

2.9-8m vs. 2.9-8f, U=88.0, p=.0404,

2.9-8m vs. 1UCNm, U=94.0, p=.0374,

2.4-8m vs. 2.4-8f,U=97.0, p=.0028.

Number of Urinations

The last Bartletts' test was applied to the number of urinations that occurred while each mouse was in the grid box. A p-value of .001 indicates that there is significant variance in amounts of urine output (Figure 8).

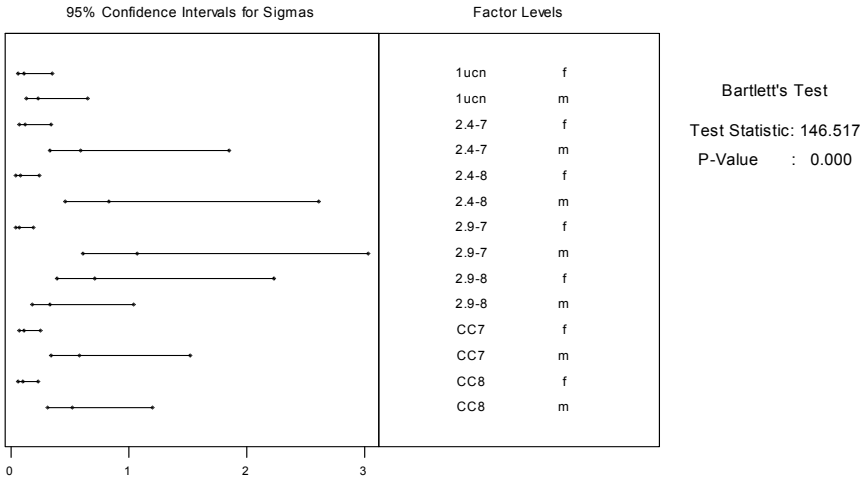

Figure 8. Test for Equal Variance for Urine Output

The median histogram for urine output also indicates a substantial difference among groups (Figure 9).

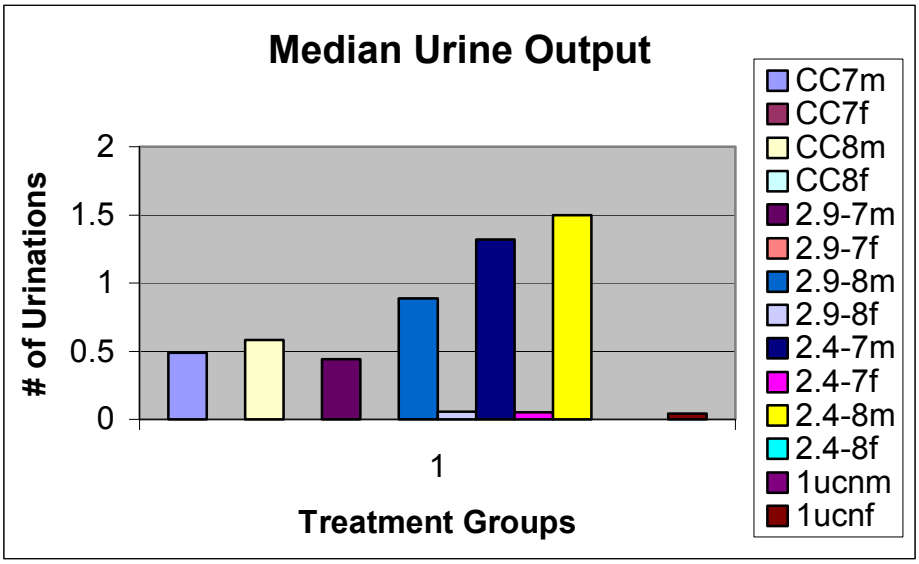

Figure 9. Median Histogram For Urine Output per Group

As with the bolus output, the Kruskall-Wallis test indicated significant differences in treatment H=12.21, F=6, p= .047 and between the sexes H=40.50, F=6, p= .001. The Mann-Whitney test was again applied to determine where the differences occurred between the groups. Significant differences occurred between the following groups:

CC7m vs. CC7f, U=161.0, p= .0016,

CC7m vs. 1 UCNm, U=130.5, p= .0118,

CC8m vs. CC8f, U=189.0, p= .0174,

CC8m vs. 2.4-8m, U=95.0, p=.0180,

2.9-8m vs. 2.9-8f, U=92, p=.0128,

2.9-8m vs. 1 UCNm, U=106.5, p=.001.

CHAPTER 4

DISCUSSION

The purpose of this study was to determine whether or not exposure through the mother to ethyl alcohol on gestational day 7 or 8 would produce detectable behavioral effects on the offspring, and whether these effects are related to the dose of the alcohol. GD-7 and GD-8 were chosen because early work indicated histological differences and damage due to prenatal ethanol exposure, namely, the forebrain in offspring exposed on GD-7 and the hindbrain on GD-8 (Sulik et al. 1981, Schambra et al. 1990, Kotch and Sulik 1992). Therefore, we hypothesized that ethanol exposure on Gestational day 7 and 8 would result in behavioral differences.

Two findings emerged from this study. First, in C57BL/6J mice, acute exposure to ethanol early in pregnancy does have some significant influence on putative measures of emotionality accessed in a novel environment. Second, there are consistent sex differences in the behaviors observed in this study regardless of prenatal exposure to alcohol.

There were 2 areas of concern involving the control groups: (1) The effect of the process of intubation on the offspring of the mice and (2) the effect of handling and neonatal testing on adult behavior of the offspring. The intubation process did not have an effect on the offsprings' behavior and there were no differences between the UC group and the GC-7 or GC-8 groups. Because there was no effect seen by the intubation process, the combined control groups (CC-7 and CC-8) were formed. Handling was the other factor considered with the control groups. For the variable of handled versus non-handled, there were no

significant differences between the UC group and the UC-N group. This could indicate that handling did not affect the mouse's response to a novel environment nor was their activity level affected in any way by previous neonatal testing.

As indicated by Avgustinovich, D, et al., (2000) a decrease in ambulation and exploration and an increase in defecation and urination are examples of behavioral changes indicating neurological insult. The number of lines crossed in an open field arena is the test for ambulation, rearing is regarded as a putative test for exploration and curiosity and bolus and urine output are commonly accepted measures of anxiety in rodents. While there were no significant effects produced by acute prenatal exposure to alcohol on activity and rearing, there were significant findings for fecal and urine output and, therefore, for inferred anxiety level in the C57BL/6J mice acutely exposed to alcohol during gestation.

Alcohol exposure on gestational day 7 generated significant differences in anxiety levels between treated groups and control groups. In a comparison between low dose males (Group 2.4-7) and control males (Group CC-7), the treated group was significantly higher in bolus output. For day 7, the high dose males (2.9-7) were significantly higher in bolus output than the untreated, un-handled control males (UC-NM).

The day 8 treatments produced some significant differences in anxiety levels between groups. A significant difference in urine output was found between the low dose males (2.4-8) and the control males (CC-8) with the low dose group being significantly higher in urine output.

In day 8, the high dose males (Group 2.9-8) were significantly higher in defecation than the untreated, un-handled control males (UC-NM). The same

high dose group (2.9-8) was higher in urination than the untreated, un-handled males (UC-NM).

Another aspect expected was a difference between the treatment days. Based on research previously conducted (Sulik et al. 1981, Sulik and Johnston 1983, Schambra et al. 1990), there was an expectation of discernable differences that could result in behavior between treatments on GD-7 and GD-8. However, no significant difference occurred between the different treatment days.

Day 7 and day 8 have been coined as critical times for development by other researchers. Molina and associates (1987) found that alcohol exposure on Day 8 resulted in the same level of physiological and behavioral changes as chronic exposure when mice were tested using horizontal screen, cliff aversion, righting reflex, and negative geotaxis tests. Other research has shown that alcohol exposure on gestational days 7-10 can result in similar behavioral deficits most commonly associated with FAS (Webster et al 1983).

Along with the differences found among the treatment and control groups, there were also several significant findings between the sexes. In activity, the females were higher in number of lines crossed than males in every group tested. Research indicates that females are typically more ambulatory than males in novel environments (Voikar et al 2001). In rearing, there were no sex differences observed. A study by Alonso, Damas, and Navarro (2000) resulted in a similar finding of no differences in rearing between the sexes.

The majority of the sex differences occurred in the bolus and urine outputs. In bolus, the female control groups (CC-7 and CC-8) were significantly higher than their male counterparts. For urine output, however, the reverse

occurred. The male controls were significantly higher than the female controls. This may be a result of males having more urine output due to marking of territory.

While females were higher in bolus output in the control groups, the opposite is true in the treated groups. For both low and high doses on day 8, the males were significantly higher in bolus output. In urine output, the males of the high dose 2.9-8 groups were higher in output than the females. The switch in bolus output between the controls and the treated groups could be a result of the alcohol affecting males more than females. One group of researchers conclude that the effect of prenatal alcohol exposure may be sexually mediated and result in males being more affected than females. These researchers looked at the neurological structural changes that occur in mice after ethanol exposure and concluded that males are more adversely affected than females (Zimmerberg and Scalzi 1989).

From the Bartlett's test, a clear lack of homogeneity of variance between individual pups per group was seen. This could be the result of certain pups receiving more ethanol exposure than others based on their position in the uterine horn. This would result in some offspring being very heavily affected by the ethanol exposure and some offspring being hardly affected at all. Our observation that the low dose treatment had more significant effects than the high dose treatment was unexpected. However it is possible that the high dose of alcohol could have resulted in fetal re-absorption or neonatal death

In our own research, we see a clear shift from females being higher in bolus output in control groups to males being higher in treated groups which

indicates that alcohol exposure and sex may be related to the level of disruption that occurs. It is evident from this study that alcohol exposure during certain periods of fetal development can result in some of the same behavioral changes that occur in chronic exposure. However, it is possible that GD 7 and GD 8 are not the only critical periods found in neurological development, but alcohol exposure on those days will result in behavioral deficit. Conversely, late developing Purkinje cells in the cerebellum were more susceptible to ethanol insult in the third trimester equivalent (neonatal period in the rat) and in the second trimester equivalent (GD 14 – birth in the rat). Researchers also noted that cells that were able to survive the insult of ethanol did not develop normally (West 1993). Extrapolating these results from experimental animals to humans can only mean that fetal exposure to alcohol at various times throughout gestation could result in FAS or at least FAE.

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