THE EFFECTS OF ALCOHOL AND ASPIRIN ON NEONATAL BRAIN DEVELOPMENT

A Senior Honors Thesis

By

MYRA DAWN BESHEAR

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
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Group: Cell Biology 2

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ABSTRACT

The Effects of Alcohol and Aspirin on

Neonatal Brain Development. (April 2000)

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This study focuses on the effects of alcohol and aspirin on brain development in the neonatal rat. The period of brain development of particular interest is known as the brain growth spurt. Previous studies have shown that the developing brain is particularly vulnerable to alcohol and other drugs during this period. The brain growth spurt occurs in humans in the third trimester of pregnancy, whereas in rats it occurs in early postnatal life. To extrapolate results from the rat species to the human species, the timing of the brain growth spurt must be equated.

For this reason, the neonatal rats were artificially reared from postnatal day (PD) 4 to PD 9. They were randomly assigned to one of nine experimental groups. Treatments for the eight artificially reared groups included various doses of aspirin, alone or in combination with a single dose of alcohol, or no drug treatment (gastrostomy control).

The ninth group was a suckle control group, and these animals were reared by their mother. The alcohol-treated groups received 4.5 g/kg/day of ethanol and either 0.0, 12.5, 25.0 or 50.0 mg/kg/day of aspirin in a milk formula solution. The remaining groups received 0.0 (gastrostomy control), 12.5, 25.0, or 50.0 mg/kg/day of aspirin in a milk formula solution that contained no alcohol.

Body weights were measured daily, and forebrain, cerebellum, and brainstem weights were measured on PD 9. Forebrain, cerebellum, and brainstem weight to body weight ratios were calculated, and a significant effect of alcohol was observed for each ratio. The brain weight to body weight ratios were significantly smaller for the alcohol-treated groups when compared to the non-alcohol-treated groups. However, a significant effect of alcohol on body weight was not observed, which indicates that the brain weight to body weight ratios were smaller for the alcohol-treated groups due to a vulnerability of the brain tissue to alcohol treatment.

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Finally, I would like to thank my advisor, Dr. James West, for providing me the opportunity to conduct undergraduate research in his laboratory and for supplying the facilities and materials necessary to complete my project.

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NOMENCLATURE

ANOVA	=	analysis of variance
BAC	=	blood alcohol concentration
FAS	=	fetal alcohol syndrome
GC	=	gastrostomy control
PD	=	postnatal day
SC	=	suckle control
SEM	=	standard error of the mean

INTRODUCTION

Many women drink alcohol during pregnancy, which can damage the developing fetus. Babies born to mothers who drink heavily during pregnancy run a substantial risk of having fetal alcohol syndrome (FAS), a pattern of damage including distinctive facial characteristics, pre- and postnatal growth deficiency, varying degrees of central nervous system dysfunction, and frequently, mental retardation. Approximately 1 to 3 out of every 1000 babies born in the United States are diagnosed with FAS (Niccols, 1994). The incidence may rise to 1 in every 300, if children who have some alcohol-related birth defects but do not exhibit all the characteristics associated with FAS were included (Harris, 1995). As a result, alcohol is a leading environmental and preventable cause of mental retardation in the Western world (Clarren and Smith, 1978; Abel and Sokol, 1987).

Unfortunately, pregnant women in our society continue to drink, despite reports in the scientific literature of alcohol's potential teratogenicity and the Surgeon General's warning (1981) advising pregnant women to abstain from drinking alcohol. Subsequently, it is important to identify and study any risk factors that may influence alcohol's effect on the developing brain. One potential risk factor is polydrug use. The concurrent use of alcohol and other drugs may increase the risk of producing more extensive damage than that which would have occurred with alcohol use alone. For

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This thesis follows the style and format of Alcoholism: Clinical and Experimental Research.

example, clinical reports provide evidence of a link between drinking and smoking (Istvan and Matazzaro, 1984; Schiffman et al.,1994), an example of polydrug use involving alcohol and nicotine. Recently, Chen and his coworkers (1998, 1999) used an animal model system to find that exposure to both alcohol and nicotine resulted in a higher mortality rate when compared to alcohol or nicotine exposure alone.

Another example of polydrug use is the ingestion of alcohol and aspirin. Several clinical and experimental justifications exist for investigating a possible interaction between alcohol and aspirin on brain growth restrictions. Clinically, it is estimated that at some point in their pregnancy, 50% of expectant mothers ingest aspirin (Hill et al., 1977; Collins, 1981; Streissguth et al., 1987). In addition, the ingestion of aspirin is often related to alcohol consumption, since individuals commonly use aspirin to treat and prevent alcohol-induced "hangovers."

Experimentally, a justification for studying an interaction between these two drugs is the discovery of several common characteristics: both cross the placenta and circulate in fetal blood (Mann et al., 1975; Goldstein, 1983), both can be toxic to cells (Klein, et al., 1981; West et al., 1986), and both alter prostaglandin levels (Hill et al., 1977; Anton and Randall, 1987). Furthermore, studies have demonstrated that both alcohol and aspirin have teratogenic potential in laboratory animals (Chernoff, 1980; Rockwood and Riley, 1985) and in humans (Zierler and Rothman, 1985; Streissguth et al., 1987). Of the multiple teratogenic effects that can result from ingestion of either of these drugs, microencephaly (a small brain for body size) and mental retardation are among the most damaging effects of either substance (Guy and Sucheston, 1986;

Bonthius and West, 1988b). Finally, animal studies have demonstrated that aspirin antagonizes some of the behavioral effects of alcohol (George and Collins, 1985).

Few studies have been published involving the investigation of an alcohol-aspirin interaction, but discrepancies exist in the available scientific literature. In some studies, aspirin appeared to protect the fetus from the deleterious effects of alcohol exposure. Specifically, Randall and her co-workers found in two separate studies (1984, 1987) that aspirin pretreatment of C57BL/6J mice exposed to alcohol on gestation day 10 reduced the number of malformed pups and prevented the increase in prenatal mortality induced by alcohol.

In contrast, other studies have demonstrated that aspirin does not offer a protective effect against alcohol-induced damage to the fetus. In fact, these studies suggest that aspirin may worsen some of the teratogenic effects of alcohol. First, Guy and Sucheston (1986), working with a different strain of mouse and at a slightly earlier stage of gestation, found a synergistic effect of aspirin and alcohol, producing both external and visceral malformations. Furthermore, they failed to observe a protective effect of aspirin in reducing alcohol-induced prenatal mortality. In a separate study, Bonthius and West (1988a) worked with neonatal rats to find that aspirin did not have a protective effect on alcohol-induced microencephaly, but instead could worsen it.

The focus of this project is to investigate the effects of alcohol and aspirin on the developing brain using a neonatal rat model system. Rodent model systems have been used extensively in the research laboratory to examine the effects of alcohol on the developing brain. The period of brain development of particular interest to researchers

is known as the brain growth spurt. During this period, the brain appears to be vulnerable to alcohol (Bonthius and West, 1990) and other drugs (Yanai and Waknin, 1985). However, brain development at birth in rats is less complete than in humans (West, 1987), so the brain growth spurt that occurs during the third trimester of human development takes place postnatally in rats (Dobbing and Sands, 1979). Consequently, to accurately model the effects of alcohol and aspirin on human brain development that occurs during the third trimester, the rats in this study were exposed to alcohol and aspirin during early postnatal life.

In order to study the effects of alcohol and aspirin on the developing brain, rats are typically placed in an artificial rearing apparatus and reared from PD 4 through PD 9. This paradigm allows the experimenter to precisely control the administration of alcohol and aspirin in a nutritionally adequate milk formula diet. Rats are usually fed 12 times daily via a surgically implanted tube, and the drugs of interest are given in a binge-type manner, since clinical studies have indicated that most women who consume alcohol during the third trimester drink heavily in short episodes, practicing a binge-drinking pattern (Stephens, 1985).

In the present study, the hypothesis being tested is that the concurrent exposure of alcohol and aspirin will result in smaller brain weights than the exposure to alcohol or aspirin alone. It would be useful to know if polydrug use of alcohol and aspirin has an effect on alcohol-induced fetal brain damage, and further, if an interaction is found, it would be of considerable benefit to know whether aspirin protects or further damages

the developing brain. This information could be employed to help minimize the devastating effects of FAS.

METHODS AND MATERIALS

SUBJECTS

The subjects were 59 male and female Sprague-Dawley rats derived from 15 different litters. The day that the dams were detected to be sperm-positive was defined as gestational day 0. The rat pups were born on gestational day 22, and that day was defined as PD 0. On PD 1, each litter was culled to 10 pups. On PD 4, the rat pups were randomly assigned to one of eight artificially reared treatment groups. A detailed list of the treatment groups and the number of animals assigned to each group is shown in Table 1. A ninth group, the suckle control (SC) group, was included for comparison with the artificially reared gastrostomy control (GC) group to assess the effect of the artificial rearing procedure. No more than two pups from each litter were assigned to the same treatment group to lower the potential confound of litter effects.

Table 1. Treatment Groups

Alcohol Dose (g/kg/day)	Aspirin Dose (mg/kg/day)	*n*
(SC) 0.0	0.0	10
(GC) 0.0	0.0	6
0.0	12.5	5
0.0	25.0	6
0.0	50.0	6
4.5	0.0	7
4.5	12.5	6
4.5	25.0	7
4.5	50.0	6

(SC), suckle control; (GC), gastrostomy control

ARTIFICIAL REARING PROCEDURE

Gastrostomy surgery

On PD 4, the rat pups were anesthetized with Metofane ®. A PE-10 gastrostomy feeding tube was press fit to a plastic-covered wire, which was lubricated and inserted into the mouth. The plastic-covered wire was carefully guided through the esophagus into the stomach and punctured through the skin to exteriorize on the lateral aspect of the pup. The wire and attached tube were drawn through the skin to the outside of the body until only the flange of the tube remained inside the stomach. The plastic-covered wire was detached from the feeding tube. To prevent the tube from being pulled out, it was anchored to the nape of the pup's neck by PE-50 washers that slipped over the gastrostomy tube.

Artificial-rearing apparatus

The apparatus is constructed of a 10-gallon glass aquarium filled with water. The water is continuously aerated with bubbles, and water temperature is maintained at 37°C to provide warmth for the animals. The top of the aquarium is covered with a sheet of plexiglass, which has 8 holes cut in it. A plastic cup fits in each hole and the bottom of the cup floats in the water. Plexiglass covers enclose the cups to keep the humidity level and temperature constant. The cup is filled with wood chip bedding, and it is partially lined with synthetic fur to simulate the presence of the mother. After gastrostomy tubes were implanted on PD 4, one rat pup was placed in each of the 8 cups. The eight pups were maintained in the artificial-rearing apparatus from PD 4 to PD 9.

Daily maintenance procedures included bathing and weighing the pups and replenishing the diet. A 12/12 hour light/dark cycle was maintained in the laboratory.

Feedings

Each day, the rat pups were fed a volume of milk formula equivalent to 33% of the mean total body weight of the litter, which was determined daily. The animals were fed every 2 hours and received 12 feedings per day, with each feeding lasting 20 minutes. The pups received the feedings through PE-50 tubing that was press-fit to the implanted gastrostomy feeding tubes. The PE-50 tubing was attached to diet-filled syringes that were activated by a timer-operated infusion pump (Harvard Apparatus). All eight artificially reared groups received a nutritionally adequate milk formula diet in each feeding.

In addition to the milk formula diet, four of the eight artificially reared groups received 4.5 g/kg/day of ethanol and 0.0, 12.5, 25.0, or 50.0 mg/kg/day of acetylsalicylic acid (aspirin) delivered as a milk solution in two consecutive feedings. The other four artificially reared groups received 0.0 (GC), 12.5, 25.0, or 50.0 mg/kg/day of aspirin in two consecutive feedings in a milk solution free of alcohol. Maltose-dextrin was added to the milk solution in place of the alcohol so that the milk solution was isocaloric to the alcohol-containing solutions.

BLOOD ALCOHOL CONCENTRATION (BAC)

To measure BAC, tail blood samples were taken from each animal on PD 6 ninety minutes after the last alcohol-containing feeding. The blood samples were taken at this particular time point because it has been shown that the peak BAC occurs approximately at this time in animals exposed to alcohol through the methods used in this study (Kelly, et al., 1987).

To obtain the tail blood samples, each rat's tail was clipped with a single-edged razor blade. A 20 µl blood sample was collected in a heparinized disposable pipette. The blood sample from each animal in the alcohol-treated groups was immediately evacuated into a gas chromatography vial, which contained 200 µl of a solution of .6 N perchloric acid and 4 mM n-propanol. The blood samples from the animals in the remaining groups were discarded. BAC was determined for the animals in the alcohol-treated groups using a gas chromatograph (Varian, Model 3400).

BRAIN MEASUREMENTS

On PD 9, each pup was removed from the artificial rearing apparatus after the last alcohol-containing feeding. The pup was then deeply anesthetized with Metofane ® and perfused intracardially with saline followed by 4% (w/v) paraformaldehyde (in 0.1M phosphate buffer, pH 7.4). After fixation, the brain was removed and separated from the spinal cord. The brain was then systematically dissected into 3 parts: forebrain, cerebellum, and brainstem, and each part was weighed.

STATISTICAL ANALYSIS

Brain weights, body weights, and brain weight to body weight ratios were analyzed using two-way analyses of variance (ANOVAs) with alcohol treatment and aspirin treatment as grouping factors. Analyses comparing the SC and GC groups and BAC analyses were conducted using one-way ANOVAs with experimental treatment as the independent factor. The α level for all analyses was set at 0.05, so significant results in this study had p-values ≤ 0.05. All statistical analyses were performed using StatView 5.0 (SPSS, Inc.).

RESULTS

BRAIN WEIGHTS

As shown in Table 2, mean forebrain, cerebellum, and brainstem weights were similar among the artificially reared groups. Three separate two-way ANOVAs were conducted to analyze the forebrain, cerebellum, and brainstem weights. No main effects of alcohol or aspirin or significant interactions were obtained from these analyses.

Three separate one-way ANOVAs were conducted to compare the GC and SC groups, and the results revealed significant differences in forebrain weight, $F(1,14) = 8.9$, $p<0.01$, and cerebellum weight, $F(1,14) = 15.2$, $p<0.01$. The SC group had significantly larger weights than the GC group. No significant difference was observed for brainstem weights.

Table 2. Mean Brain Region Weights of Nine-Day Old Rat Pups Exposed to Different Daily Doses of Alcohol and Aspirin

Alcohol Dose (g/kg/day)	Aspirin Dose (mg/kg/day)	Forebrain Weight (g)	Cerebellum Weight (g)	Brainstem Weight (g)
0.0	0.0	0.532 ± 0.016	0.050 ± 0.002	0.049 ± 0.003
0.0	12.5	0.498 ± 0.041	0.044 ± 0.006	0.045 ± 0.004
0.0	25.0	0.494 ± 0.011	0.048 ± 0.001	0.046 ± 0.003
0.0	50.0	0.509 ± 0.019	0.048 ± 0.003	0.047 ± 0.003
4.5	0.0	0.503 ± 0.013	0.045 ± 0.002	0.043 ± 0.003
4.5	12.5	0.495 ± 0.016	0.047 ± 0.003	0.046 ± 0.003
4.5	25.0	0.497 ± 0.013	0.045 ± 0.002	0.044 ± 0.002
4.5	50.0	0.466 ± 0.009	0.043 ± 0.002	0.043 ± 0.002

Values represent means ± standard error of the mean (SEM).

BODY WEIGHTS

As indicated in Figure 1, the artificially reared groups had similar mean daily body weights throughout the artificial rearing period. A three-way ANOVA performed on body weight gain from PD 4 to PD 9 revealed no main effects of alcohol or aspirin or significant alcohol×aspirin interactions.

A one-way ANOVA conducted to compare the GC and SC groups revealed that body weight gain from PD 4 to PD 9 was significantly lower for the GC group, $F(1,14) = 157.5$, $p<0.0001$. This difference indicates that the artificial rearing procedure affected body weight gain.

BRAIN WEIGHT TO BODY WEIGHT RATIOS

Forebrain weight, cerebellum weight, and brainstem weight to body weight ratios were calculated for each group [(brain region weight/PD 9 body weight) × 100]. As shown in Figure 2, treatment with 4.5 g/kg/day of alcohol resulted in smaller ratios for the forebrain, cerebellum, and brainstem. Three separate two-way ANOVAs were conducted on the ratios, and a significant main effect of alcohol was revealed on the forebrain weight to body weight ratio, $F(1,41) =20.9$, $p<0.0001$, the cerebellum weight to body weight ratio, $F(1,41) = 21.9$, $p<0.0001$, and the brainstem weight to body weight ratio, $F(1,41) = 11.8$, $p<0.01$. However, no significant effects of aspirin or the interaction of alcohol and aspirin were found.

Three separate one-way ANOVAs were performed to compare the forebrain weight, cerebellum weight, and brainstem weight to body weight ratios of the GC and

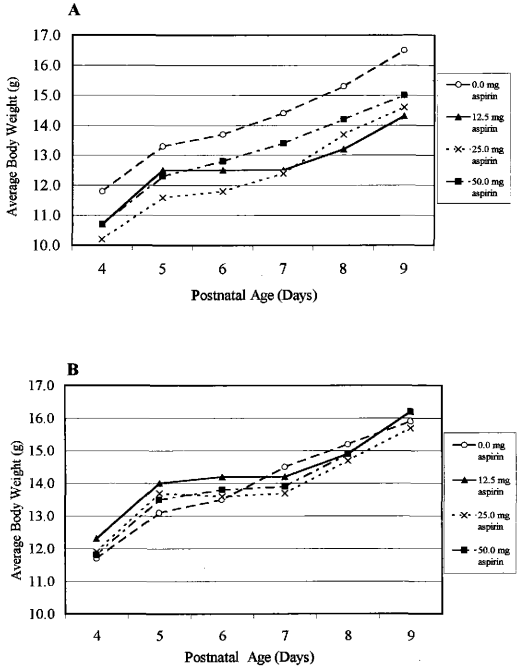

Figure 1. Average Daily Body Weights of Artificially Reared Rat Pups Exposed to Alcohol and/or Aspirin. (A) Nonalcohol-treated groups. (B) Alcohol-treated groups.

SC groups. Significant differences were found for all the analyses. The GC group ratios were significantly smaller when compared with SC group ratios for the forebrain to body weight ratio, $F(1,14) = 14.4$, $p<0.01$, and the cerebellum to body weight ratio, $F(1,14) = 8.1$, $p<0.05$. However, brainstem to body weight ratios for the GC group were significantly larger when compared with the SC group, $F(1,14) = 9.1$, $p<0.01$.

BLOOD ALCOHOL CONCENTRATIONS

As shown in Table 3, rat pups in the alcohol-treated groups had similar peak BACs. A one-way ANOVA revealed no significant effect of aspirin on peak BAC.

Table 3. Peak Blood Alcohol Concentrations on Postnatal Day 6

Aspirin Dose (mg/kg/day)	Blood Alcohol Concentration (mg/dl)
0.0	229.1 ± 34.8
12.5	180.8 ± 52.9
25.0	201.0 ± 37.1
50.0	231.8 ± 40.5

Values represent means ± SEM.

CONCLUSION

In this study we tested the hypothesis that concurrent administration of alcohol and aspirin would significantly reduce brain weights in comparison to alcohol or aspirin treatment alone. No significant interactions of alcohol and aspirin were observed, but alcohol was found to significantly reduce brain weight to body weight ratios.

In the present study, alcohol had a subtle effect on brain weight reduction. Alcohol treatment alone did not significantly reduce forebrain, cerebellum, or brainstem weights. In a previous study conducted by Bonthius and West (1988a) using the same experimental paradigm, alcohol treatment was shown to significantly reduce cerebellum, brainstem, and total brain weights, and brain weight to body weight ratios. However, the present study differed from that of Bonthius and West in one important respect: the alcohol dose used in this study (4.5 g/kg/day) was smaller than the dose used by Bonthius and West (6.6 g/kg/day). The smaller dose used in this study may not have been large enough to produce an interaction with aspirin to reduce brain weights.

Another difference between the current study and the previous study is the sample size. In the study by Bonthius and West, 120 animals were studied (1988a), whereas only 60 animals were studied in the present experiment. The fact that brain weight to body weight ratios were significantly reduced by alcohol but brain weights alone were not could possibly be attributed to the smaller sample size.

Though the alcohol-induced effect was subtle in the present study, the results are still of clinical importance. The results of the current study indicate that smaller doses of

alcohol and lower BACs can have an effect on brain development, which suggests that pregnant women should use caution even when drinking small amounts of alcohol in order to protect the developing fetus from alcohol-induced brain damage.

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VITA

Myra Dawn Beshear was born in Ada, Oklahoma in 1978 and moved to Woodward, Oklahoma in 1990, where she graduated from Woodward High School in 1996. She moved to Texas in September 1996 to attend Texas A&M University, where she was awarded the President's Endowed Scholarship. She will graduate magna cum laude in May 2000 with a B.S. in biomedical science and both University and Foundation Honors degrees. Following graduation, she plans to attend the University of Texas College of Pharmacy in Austin, Texas, beginning in the fall 2000 semester, to pursue a Doctor of Pharmacy degree. Her permanent address is Route 1 # 22, Woodward, OK 73801.