An Investigation of the Effects of Acute vs. Chronic Ethanol Exposure on the Embryonic Chick Brain:

A Model of Fetal Alcohol Syndrome-Associated Brain Damage

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I. Abstract

The chick embryo was used as a model for Fetal Alcohol Syndrome (FAS) to perform a dose-response study of brain damage. Two common maternal drinking patterns were compared. Chronic drinking was simulated by administering 2 μl doses of 0.5% ethanol eight times during the development of the embryo. Acute drinking was simulated by administering 5 μl doses of 0.5% ethanol three times during development. Significant differences ($p < 0.01$) were observed between the two treatment groups with embryos in the acute group manifesting considerably more brain damage (as determined by brain weight and brain weight-to-body weight ratio measurements) than embryos in the chronic treatment group. This result indicates that the amount of brain damage incurred as a result of alcohol consumption is a function of blood alcohol concentration levels. No significant differences ($p > 0.01$) were observed among the treatment groups in neuron counts from the right cerebral hemispheres of the brains. This result indicates that the mechanism of FAS-related CNS damage does not occur through the interference of alcohol with cell division.

II. Introduction

Exposure to ethanol in utero results in a variety of physical and behavioral deficits commonly termed the Fetal Alcohol Syndrome (FAS). FAS was first described in 1973 and is characterized by nervous system dysfunction, sub-normal mental capacity, growth deficiencies and various physical malformations, the most notable of which is atypical facial appearance (Rosell, 1974). The single most common defect observed in all models of FAS is fetal growth retardation (Pennington, 1957). Previous research in this field has focused on the molecular mechanisms responsible for alcohol-induced growth deficits. However, studies are necessary for establishing which drinking patterns and dose levels are most harmful to the developing fetus. This investigation will attempt to determine which of two common maternal alcohol consumption patterns, acute drinking (heavy doses of alcohol interspersed by long time intervals) or chronic drinking (small doses of alcohol consumed in short, regular time intervals), is more harmful to fetal brain growth.

Suppressed brain growth in fetuses is very tragic, as children that suffer from this in utero are often born with a variety of brain dysfunctions, including, most commonly, mental retardation, hyperactivity and microencephaly (Bonthius, 1991). Although studies have only followed children diagnosed with FAS until their tenth year, it is likely that the brain dysfunctions that manifest due to the disease persist well beyond this time period and are

likely permanent.

One of the most important and frequently asked questions regarding FAS is "How much alcohol is harmful to the developing fetus?" The answer to this seemingly simple question is, in fact, quite complex due to factors such as differences in genetic susceptibility (Chernoff, 1980), competence in alcohol metabolism (Pennington, 1987), timing of the alcohol insult (West, 1987), and concomitant ingestion of other drugs (Bonthius, 1989).

One additional factor that may influence the risk and severity of alcohol-related birth defects is the pattern of alcohol consumption. Since little is known about the pattern-related effects of alcohol consumption on brain development in the fetus, the purpose of this investigation will be to determine an answer to this question.

There have been many published studies which indicate that exposure to ethanol in utero causes suppressed brain growth. For example, Pennington and Kalmus (1987) reported a 27% reduction in the brain weight of chick embryos after a single treatment of a 1 g/kg dose of ethanol at the start of incubation (day 0) as compared to controls receiving the same dose of chick Ringer's solution. In 1984, Boyd et al. reported that single dose treatments of 5 day chick embryos with 200 μl of a solution of ethanol (65 mg of ethanol /100 g egg) resulted in a significant inhibition of brain growth (an 11% reduction as compared to controls) measured on day 10.

A very convenient measure of alcohol-related brain damage is the degree of microencephaly induced. There have been several

studies published that indicate a strong and direct correlation between degree of brain damage and degree of microencephaly. Sterling et al. (1990) administered weekly doses of ethanol to gravid pigtailed macaques (Macaca nemestrina). The infants born were then assessed for abnormalities in physical and behavioral development at six months of age. All infants that were exposed to enough ethanol in utero to manifest retarded mental and behavioral development characteristic of brain damage also exhibited marked microencephaly. The infants that were not exposed to enough ethanol in utero to manifest any mental and behavioral retardation did not suffer from microencephaly. They also determined that the more severe the mental and behavioral retardation there was, the more microencephaly there was. Because of evidence that has been obtained in studies like this one, this investigation will measure microencephaly (brain weight) as one indicator of brain damage.

In addition to the determination of the degree of brain growth suppression by simply measuring the brain weight, the degree of brain growth suppression has also been determined by measuring brain cyclic AMP and tissue prostaglandin E2 (PGE2) levels. Pennington (1982) reported that ethanol inhibits chick brain growth via a mechanism that involves increased synthesis of cyclic AMP secondary to increases in tissue levels of PEG2. The more brain cyclic AMP and tissue PGE2 found, the more brain damage was found to have occurred. In the Pennington (1982) study, PGE2 levels and cyclic AMP levels were assayed in perchloric acid extracts of tissues and brain by the use of ^{131}I-RIA kits. It is not feasible to use such an assay in this investigation due to the medical

hazards of ^{131}I and time constraints that would most likely prevent the generation of an accurate standard curve.

However, there have been histological investigations that have yielded evidence of ethanol-induced brain growth suppression on the cellular level. Bonthius and West (1991) observed ethanol-induced Purkinje cell and granule cell losses in the cerebellum of the fetal rat. Since mental retardation includes loss of cortical competence, the determination of the extent of brain damage in this investigation will rely on measurements of microencephaly corroborated by estimates of total cell density in the cerebrum.

Animal research on alcohol-induced brain damage has been essential in understanding alcohol-related developmental disabilities in humans. For this study, the chick embryo will serve as an animal model for FAS for two reasons. First, the chorioallantoic membrane (CAM), which surrounds the chick embryo and is formed from the chorion and the fused allantoic sac, is analogous to the placenta of the human embryo (Fig. 1). By applying ethanol directly to the CAM, the material variables which can affect ethanol metabolism prior to exposure to the fetal brain will be eliminated. Secondly, the brain damage that the chick embryo will suffer can correlate with the brain damage that a human embryo would suffer if subjected to the same relative protocol. Since the three week gestation period of the chick mimics the three trimesters of human pregnancy, the results from studies on the chick can be extrapolated to humans.

It is now commonly known that alcohol is teratogenic and that it is especially harmful to the brain of the embryo. Further

experimentation in this particular field is important in that the evidence obtained may serve as a guide for clinical observations in humans and yield information essential for a rational approach to intervention in populations at risk for alcohol-related birth defects.

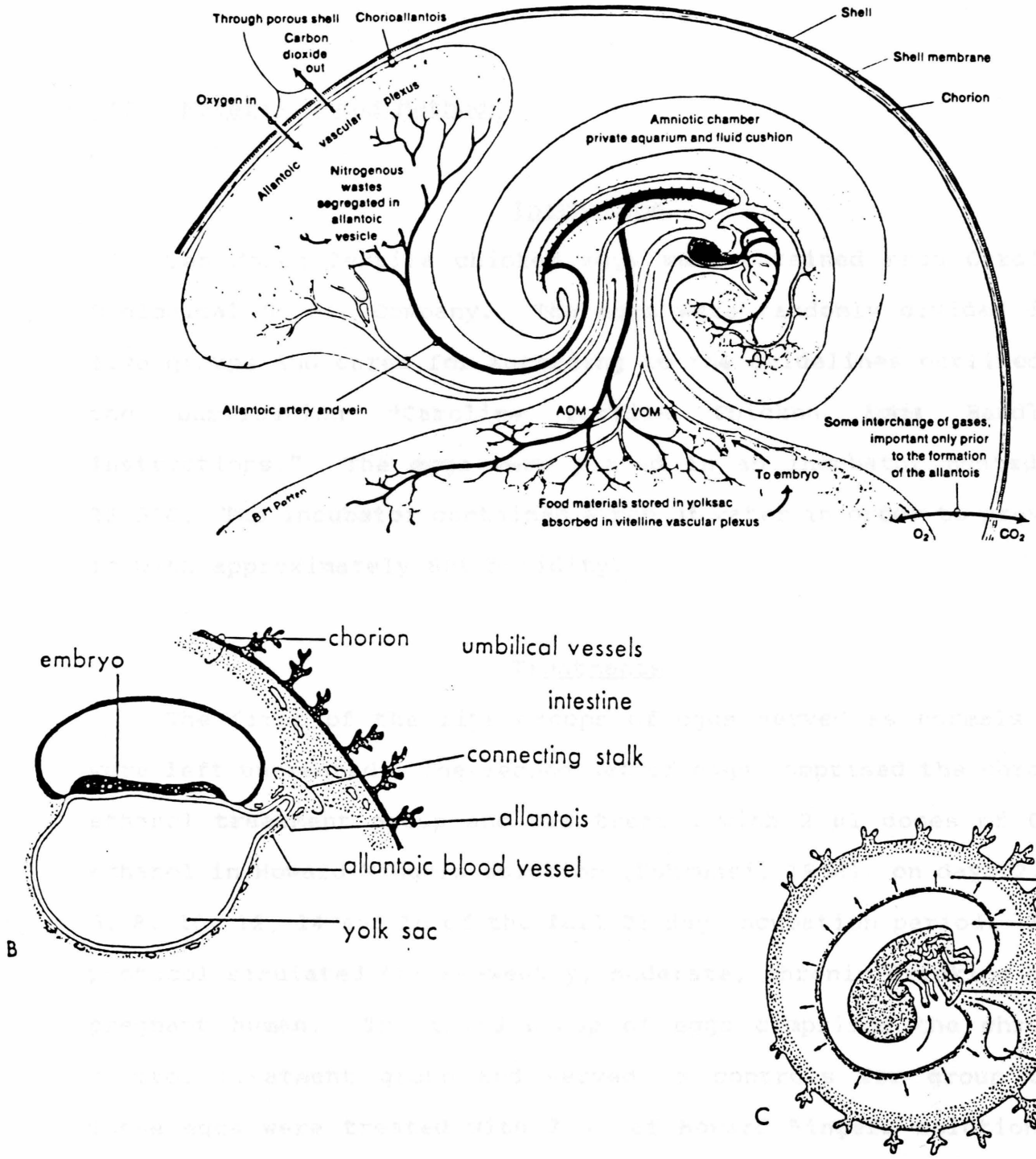

Fig. 1: Diagrams illustrating the homology between the chick chorioallantois and the human placenta. A) 4-day chick embryo; B) Three week human embryo; C) Ten week human embryo. Abbreviations: AOM, omphalomesenteric (vitelline) artery; VOM, omphalomesenteric (vitelline) vein. (Mathews, 1987)

III. Materials and Methods

Incubation

Ten dozen fertile chicken eggs were obtained from Carolina Biological Supply Company. The eggs were randomly divided into five groups and cared for according to the guidelines outlined in the publication "Carolina Fertile Chicken Eggs Handling Instructions." The eggs were placed in an incubator heated to 38.5°C. The incubator contained a pan of water in order to provide it with approximately 80% humidity.

Treatments

The first of the five groups of eggs served as normals and were left untreated. The second set of eggs comprised the chronic ethanol treatment group and was treated with 2 μl doses of 0.5% ethanol in Howard Ringers solution (Ephrussi, 1936) on days 2, 4, 6, 8, 10, 12, 14 and 16 of the full 21 day incubation period. This protocol simulated thrice-weekly, moderate, chronic drinking by a pregnant human. The third group of eggs comprised the chronic control treatment group and served as controls for group two. These eggs were treated with 2 μl of Howard Ringers solution on days 2, 4, 6, 8, 10, 12, 14 and 16 of incubation. The fourth group of eggs comprised the acute treatment group and received 5 μl doses of 0.5% ethanol in Howard Ringers solution on days 2, 9 and 16. This protocol simulates heavy, binge drinking by a pregnant human. The fifth and final group served as controls for the fourth group

and received 5 μl doses of Howard Ringers solution on days 2, 9 and 16. This group comprised the acute control treatment group. Table 1 summarizes the treatments received by the chick embryos in each of the five groups.

The solutions were applied to the chorioallantoic membrane (CAM) (Fig. 1) using a sterilized Hamilton syringe inserted through a small hole in the eggshell directly above the embryo. The location of the embryo was determined on the second day of incubation based on its shadow as seen when illuminated from below. The surface of the egg at the air sac was sterilized and a sterile dissecting needle was used to puncture the eggshell in order to release the air. This allowed the embryo to be lowered out of range prior to drilling the second hole. Both holes were covered with square pieces of scotch tape to prevent the entry of contaminated air. The tape on the second hole was removed for each treatment application and subsequently reapplied. The drilling of holes in the egg shells and all treatment applications were done in a laminar flow hood, using equipment sterilized with 70% ethanol.

Body and Brain Weight Measurements

On the nineteenth day of incubation, all chicks were removed from their shells and sacrificed by decapitation. Only the chicks that were alive upon removal from their shells were dissected. Immediately following decapitation, each embryo was weighed to the nearest hundredth of a gram on an analytical balance. After weighing each entire embryo, the brains were removed in their entireties from the skulls and separated from the spinal cords.

The brains were weighed with the same accuracy as the entire embryos using the same balance. After weighing, the bodies of the embryos were discarded and the brains were placed in individually numbered jars of Bouin's fixative until histological studies could be performed.

Histological Analysis

A total of ten brains (two from each treatment group) were studied histologically. Each brain was removed from Bouin's fixative and placed in 25% formalin for 24 hours. The brains were then removed from the formalin, wrapped in individual pieces of gauze and placed under running tap water for 24 hours. The brains were embedded in fresh egg yolk and placed in a chamber containing 25% formalin for 72 hours. The embedded brains were then placed for 24 hours in a 10% formalin chamber and finally overnight in a chamber containing a formalin and sucrose solution.

Two μm mid-sagittal sections of the right cerebral hemisphere of each brain were prepared using a Reicher-Jung Ultracut E microtome. Care was taken to ensure that the sections of each brain were taken from approximately the same location within each cerebrum. The sections were mounted onto gelatin-coated glass slides and were air-dried. The sections were then dehydrated through a graded series of alcohols. The sections were stained with cresyl violet, differentiated, dehydrated, cleared in xylene and coverslipped.

Neuronal populations were counted from single sections from the right cerebrum of each brain using a Leitz Labrolux 12

microscope. A cell was included in the count only when it possessed a clearly discernible nuclear membrane. Neuron counts from two separate 3.75×10^{-2} mm^2 areas of each slide were obtained. This area was determined by dividing the area of a rectangular field of view defined by a particular eyepiece by the total magnification. The field of view was 15 mm^2 and the total magnification was 400x. Continuous counts were made of each area until two consecutive counts were the same. The identifying numbers on each slide were covered prior to performing the counts to ensure an analysis blind to the treatment group.

Statistical Analysis

Tests of significance were conducted using a "t-test" program from Graph (San Jose Software) on a HP Model 86 computer with $p < 0.01$ as the standard of significance.

Treatment Group	Treatment
Chronic Ethanol	2 μl 5% ethanol in Howard Ringers days 2, 4, 6, 8, 10, 12, 14, 16
Chronic Control	2 μl Howard Ringers days 2, 4, 6, 8, 10, 12, 14, 16
Acute Ethanol	5 μl 5% ethanol in Howard Ringers days 2, 9, 16
Acute Control	5 μl Howard Ringers days 2, 9, 16
Normal	untreated

Table 1: The five treatment groups and the treatments the chick embryos in each group received during the 19 day incubation period.

IV. Results

The mortality rate for the embryos in the normal treatment group was much lower than 30-40% which is, according to Carolina Biological Supply, normal for incubated eggs (Carolina, 1989). The mortality rates for the embryos in the manipulated treatment groups were higher, with only one group exceeding 40% (Table 2).

Tables 3-7 show total body weight, brain weight and brain weight-to-body weight ratios for the embryos in each treatment group upon sacrifice at day 19.

Table 8 shows the mean values for each of these three properties for the embryos in the acute control and acute ethanol treatment groups. This Table also shows the results of a t-test analysis of these means when comparing the two groups. As shown in this Table, the mean total body weight and mean brain weight were found to be significantly smaller in the acute ethanol group. No significant difference was demonstrated between the two groups in mean brain weight-to-body weight ratios.

Table 9 compares the same three mean values for the embryos in the chronic control and chronic ethanol treatment groups. A t-test demonstrated a significantly smaller brain weight in the chronic ethanol treatment group. None of the other differences were found to be significant at the 95% level.

In Table 10, the acute ethanol and chronic ethanol treatment groups are compared for mean total body weight, mean brain weight and mean brain weight-to-body weight ratios. T-tests demonstrated

significant differences between the two groups in both mean brain weight and mean brain weight-to-body weight ratios. The embryos in the acute ethanol treatment groups had significantly lower values for both properties. No significant difference was found when comparing the mean total body weight of the two groups.

Table 11 shows mean neuron counts for standard areas of 3.75×10^{-2} cm^2 within the right cerebral hemispheres of the examined brains. There were no significant differences found among any of the treatment groups for this property at the 95% level of confidence.

Figures 2 and 3 graphically illustrate the mean total body weight and the mean total brain weight for each of the treatment groups.

Thus, these studies demonstrate that the chicks administered acute doses of ethanol manifest a greater degree of brain damage, as determined by a reduction in brain weight, than do the chicks that received chronic doses of ethanol. The chicks in the acute treatment group did not, however, exhibit a reduction in neuronal density.

Treatment Group	Mortality Rate
Chronic Ethanol	23
Chronic Control	31
Acute Ethanol	54
Acute Control	7
Normal	0

Table 2: The mortality rates (percentage of embryos that were dead upon opening their shells on day 19) for each of the five treatment groups.

Chick Number	Total Body Weight (g.)	Brain Weight (g.)	Ratio: Brain Weight / Body Weight
1	12.15	0.53	0.044
2	11.47	0.48	0.042
3	14.24	0.57	0.040
4	12.93	0.55	0.043
5	11.54	0.49	0.043
6	16.40	0.68	0.042
7	13.18	0.53	0.040
8	15.86	0.63	0.040

Table 3: Total body weight, brain weight and brain weight-to-body weight ratios for chick embryos in the chronic ethanol treatment group.

Chick Number	Total Body Weight (g.)	Brain Weight (g.)	Ratio: Brain Weight / Body Weight
1	14.01	0.64	0.046
2	19.11	0.83	0.043
3	18.98	0.81	0.043
4	18.95	0.80	0.042
5	17.63	0.74	0.042
6	10.69	0.59	0.055
7	17.45	0.68	0.039
8	16.16	0.70	0.043

Table 4: Total body weight, brain weight and brain weight-to-body weight ratios for chick embryos in the chronic control treatment group.

Chick Number	Total Body Weight (g.)	Brain Weight (g.)	Ratio: Brain Weight / Body Weight
1	12.27	0.47	0.038
2	11.14	0.43	0.039
3	13.43	0.49	0.036
4	13.95	0.49	0.035
5	13.80	0.48	0.035

Table 5: Total body weight, brain weight and brain weight-to-body weight ratios for chick embryos in the acute ethanol treatment group.

Chick Number	Total Body Weight (g.)	Brain Weight (g.)	Ratio: Brain Weight / Body Weight
1	16.50	0.73	0.044
2	16.39	0.71	0.043
3	15.92	0.68	0.043
4	17.23	0.82	0.048
5	16.36	0.79	0.048
6	15.82	0.69	0.044
7	17.10	0.80	0.047
8	14.99	0.62	0.041
9	15.93	0.68	0.043
10	16.58	0.77	0.046
11	16.39	0.70	0.043
12	16.28	0.74	0.045
13	15.89	0.67	0.042

Table 6: Total body weight, brain weight and brain weight-to body weight ratios for the chick embryos in the acute control treatment group.

Chick Number	Total Body Weight (g.)	Brain Weight (g.)	Brain Weight / Body Weight Ratio:
1	17.83	0.74	0.042
2	19.42	0.89	0.046
3	18.68	0.86	0.046
4	17.93	0.84	0.047
5	17.63	0.79	0.045

Table 7: Total body weight, brain weight and brain weight-to-body weight ratios for the unmanipulated chick embryos (normals).

Treatment Group	Mean Total Body Weight (g. ± SD)	Mean Brain Weight (g. ± SD)	Mean Brain Weight-to-Body Weight Ratios (± SD)
Acute Control	16.28 ± 0.58	0.72 ± 0.06	0.044 ± 2.26×10^{-6}
Acute Ethanol	12.82 ± 1.09	0.47 ± 0.02	0.037 ± 0.00

Table 8: Mean total body weight, mean brain weight and mean brain weight-to-body weight ratios for day 19 chick embryos in the acute control and acute ethanol treatment groups. T-tests demonstrate a significant difference between the two treatment groups in mean total body weight ($t= 9.09$; $p< 0.01$) and mean brain weight ($t= 9.12$; $p< 0.01$). No significant difference is demonstrated in mean brain weight-to-body weight ratios ($t= 1.51$; $p> 0.01$).

Treatment Group	Mean Total Body Weight (g. ± SD)	Mean Brain Weight (g. ± SD)	Mean Brain Weight-to Body Weight Ratios (± SD)
Chronic Control	16.62 ± 2.95	$0.72 \pm 2.73 \times 10^{-2}$	$0.044 \pm 1.52 \times 10^{-5}$
Chronic Ethanol	13.47 ± 1.88	0.56 ± 0.07	0.042 ± 0.00

Table 9: Mean total body weight, mean brain weight and mean brain weight-to-body weight ratios for day 19 chick embryos in the chronic control and chronic ethanol treatment groups. A t-test demonstrates a significant difference between the two treatment groups in total brain weight ($t= -4.27$; $p< 0.01$). T-test demonstrate no significant difference between the two treatment groups in mean total body weight ($t= -2.58$; $p> 0.01$) and mean brain weight-to-body weight ratios ($t= -1.33$; $p> 0.01$).

Treatment Group	Mean Total Body Weight (g. ± SD)	Mean Brain Weight (g. ± SD)	Mean Brain Weight-to-Body Weight Ratios (± SD)
Acute Ethanol	12.82 ± 1.09	0.47 ± 0.02	0.037 ± 0.00
Chronic Ethanol	13.47 ± 1.88	0.56 ± 0.07	0.042 ± 0.00

Table 10: Mean total body weight, mean brain weight and mean brain weight-to-body weight ratios for day 19 chick embryos in the acute ethanol and chronic ethanol treatment groups. T-tests demonstrate a significant difference between the two treatment groups in mean brain weight ($t= -2.76$; $p< 0.01$) and mean brain weight-to-body weight ratios ($t= -5.41$; $p< 0.01$). A t-test demonstrates no significant difference between the two treatment groups in mean total body weight ($t= -0.58$; $p> 0.01$).

Treatment Group	Mean Neuron Count (± SD)
Normals	106 ± 3.25
Chronic Control	105 ± 6.00
Chronic Ethanol	105 ± 4.10
Acute Control	104 ± 3.10
Acute Ethanol	103 ± 2.50

Table 11: Mean neuron counts taken from 3.75×10^{-2} mm^2 areas within the right cerebral hemisphere. There is no significant difference between any of the treatment groups ($p > 0.01$).

Fig. 2. Mean Total Body Weight

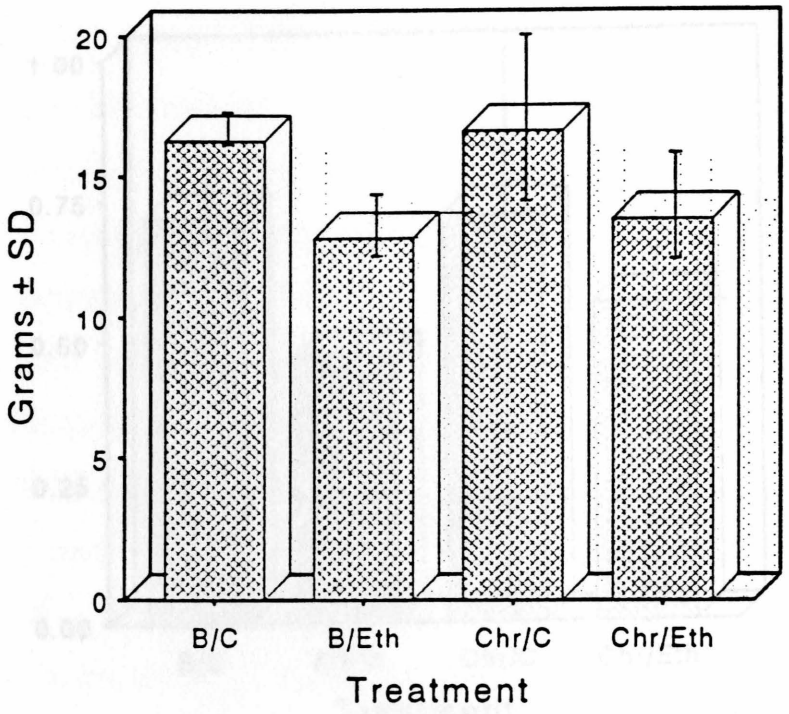

Fig. 2: Mean total body weight of day 19 chick embryos in the four manipulated treatment groups. Error bars represent standard deviations. See Tables 8, 9 and 10 for t-test results. Abbreviations: B/C, acute control treatment group; B/Eth, acute ethanol treatment group; Chr/C, chronic control treatment group; Chr/Eth, chronic ethanol treatment group.

Fig. 3. Mean Total Brain Weight

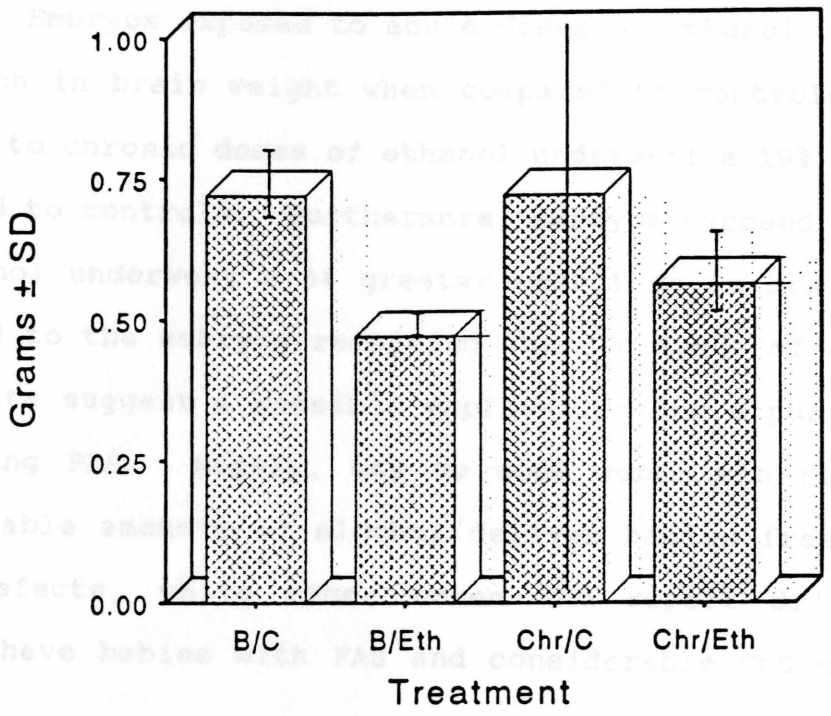

Fig. 3: Mean total brain weight of day 19 chick embryos in the four manipulated treatment groups. Error bars represent standard deviations. See Tables 8, 9 and 10 for t-test results. Abbreviations: B/C, acute control treatment group; B/Eth, acute ethanol treatment group; Chr/C, chronic control treatment group; Chr/Eth, chronic ethanol treatment group.

V. Discussion

Exposure to ethanol during the gestational development of the chick embryo resulted in a dose-dependent reduction in brain weight. Embryos exposed to acute doses of ethanol underwent a 21% reduction in brain weight when compared to controls, and embryos exposed to chronic doses of ethanol underwent a 19% reduction when compared to controls. Furthermore, embryos exposed to acute doses of ethanol underwent a 5% greater reduction in brain weight when compared to the embryos receiving chronic doses of ethanol. These data suggest a possible explanation for a puzzling question concerning FAS. Namely, why do some women who report drinking considerable amounts of alcohol deliver babies free from obvious birth defects, while other women who report drinking similar amounts have babies with FAS and considerable CNS damage (Sokol, 1980)?

One possible explanation for the increase in brain damage in the embryos receiving "binge" doses of ethanol is that they achieve a greater blood-alcohol concentration (BAC) during development. Although the acute chicks in this study received a total of 15 μl of ethanol solution during development (as compared to the chronic embryos, who received 16 μl of the ethanol solution), their total dose was divided among three separate exposures (as opposed to eight separate exposures for the chronic embryos). Thus, the chicks exposed to acute doses of ethanol manifested higher BACs than did the chicks exposed to chronic doses of ethanol. Since peak BAC is a function not only of the dose of alcohol consumed but

also of the rate at which it is consumed, this explanation seems likely. Further studies investigating the precise mechanisms which cause this ethanol-induced suppression in brain growth are indeed warranted.

A reduction in total body weight was observed in both the acute ethanol and chronic ethanol treatment groups. However, the reduction in total body weight in the chronic ethanol treatment group was not found to be significant at the 95% level of confidence. Similarly, the mean total body weight of the embryos in the acute ethanol treatment group is lower (although not significantly) than that of the embryos in the chronic ethanol treatment group. These results suggest that exposure to ethanol during gestation adversely affects brain weight to a greater extent than total body weight.

The mean brain weight-to-body weight ratio for the acute ethanol treatment group is 12% lower than that for the chronic ethanol treatment group. This indicates that the growth rates of the brains are inhibited to a greater extent than the growth rates of the total bodies for the embryos in the acute ethanol treatment group.

There was no significant difference between any of the treatment groups in mean neuronal counts. This result indicates that FAS-related CNS damage does not occur through the interference of alcohol with cell division. Therefore, it is suggested by this study that the alcohol-induced reduction in brain weight is due not to interference in the initial proliferation of neuroblasts, but in interference with neuronal differentiation, growth, and the

formation of networks.

These results may have interesting clinical and experimental implications. A smaller than normal brain is a primary feature of FAS that is commonly associated with mental retardation. The ability to restrict brain growth by manipulating the BAC without confounding the important variable of dose simplifies the task of determining the minimum amount of alcohol needed to produce teratogenic effects. Previous clinical studies have suggested that as few as two drinks per day represent a risk to the fetus (Plant, 1985). For this reason, the Surgeon General has recommended that pregnant women abstain completely from alcohol. Unfortunately, many women continue to consume some alcohol during pregnancy despite this warning. Therefore, it is important to determine safe levels of alcohol consumption during pregnancy. The evidence obtained in this study demonstrate that it is not adequate to consider a safe level of alcohol consumption merely in terms of dose. However, if drinking alcohol during pregnancy is unavoidable, chronic consumption is less harmful to the fetus than acute consumption.

In summary, the degree of microencephaly was highly correlated with maximum BAC. Therefore, the risk and severity of alcohol-induced brain damage to a developing fetus would be expected to increase for any given dose of alcohol as a consequence of conditions leading to higher BACs, including acute consumption patterns.

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