The Effects of Ethanol on Embryonic Chick Brain Mitotic Activity:

A Model for Fetal Alcohol Syndrome

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I. Abstract

Fetal Alcohol Syndrome (FAS) results from infrequent, high levels of ethanol consumption by the pregnant mother, and leads to microcephaly, growth and mental retardation, and facial disfiguration in offspring. The chick has been chosen as a model in FAS research in brain development because of the homology of the avian chorioallantoic membrane and mammalian placenta. In order to study the effects of ethanol on brain mitotic activity, a possible cause of microcephaly, ethanol's effect on tritiated thymidine incorporation into brain DNA was determined in these studies. Although measurements of brain weights support the conclusion that ethanol consumption leads to microcephaly, high brain weight/ body weight ratios suggest that overall growth inhibition may have more of a causal role in brain size. In addition, DNA percent composition and scintillation studies suggest that lack of brain mitotic activity is not the cause of microcephaly.

II. Introduction

Fetal Alcohol Syndrome (FAS), first described in 1973, includes a group of physical and behavioral abnormalities in infants born to alcoholic mothers (Gorman, 1993). Although it has been long identified and studied, many pregnant mothers are either poorly informed of the gravity of FAS's effects, or they purposefully ignore warnings with the mistaken belief that they are immune to the effects, both physically and legally. Frighteningly, "maternal alcohol abuse is thought to be *the* most common cause of mental retardation" (Moore, 1988). As a result, FAS has been termed "a national tragedy" in France which threatens to reach equal proportions worldwide (Dorozyaski, 1993). Only within the last decade have researchers and doctors begun to term high levels of prenatal alcohol consumption "fetal abuse" and to consider it a serious offense which must be stopped (Condon, 1986).

FAS is characterized by mental retardation and growth deficiency, microcephaly, ocular and joint anomalies, and facial disfiguration, including maxillary hypoplasia and abnormal palmar creases (Moore, 1988; Shoemaker *et al.*, 1983). In addition, decreased birth weight and altered learning ability have been demonstrated (Creighton-Taylor and Rudeen, 1991) as well as continued sleep disorders characterized by less REM sleep, which is necessary for the consolidation of memory (Dorozyaski, 1993). On a cellular level, decreased protein synthesis, low cerebral neurotransmitter levels, and hormonal imbalances have all been demonstrated but poorly understood (Druse *et al.*, 1986; Henderson *et al.*, 1981). Apparently, then, the head and brain are most affected developmentally by FAS, although the physiological bases and

mechanisms of these effects remain unknown (Yool and Gruol, 1987; Henderson *et al.*, 1981).

Research has begun to focus on the exact causes of FAS, such as the frequency and quantity of alcohol that produces the symptoms (Gorman, 1993), and its cellular and molecular targets (Michaelis and Michaelis, 1994). Previous and ongoing studies using rats or mice as a model have focused on the neurology and molecular mechanisms responsible for the observed effects, most notably mental and growth retardation (Miller, 1988), head and face malformation (Hogan and Barnes, 1992), and altered cerebral neurotransmitter balance (Druse et al, 1986; Henderson *et al.*, 1981).

In a related study using the chick as a model, a comparison was made between effects of "binge" versus "chronic" alcohol exposure during development. It was found that brain weight and size were most affected by infrequent, periodic "binge" drinking of large amounts of alcohol associated with alcoholism (Gorman, 1993). These studies cause us to question the exact nature of brain growth retardation to see if there is a particular mechanism which could be stopped or prevented. Kandel and Schwartz (1981) describe the developmental process of the embryonic brain in terms of cell processes. Once the neural tube has fused, a simple layer of epithelial stem cells begins to rapidly divide and form several layers of nuclei within the epithelium. These nuclei must migrate to the ventricular surface before dividing again, but cell division there is limited to only a few cycles. The postmitotic cells must then migrate to their appropriate zones of the brain as predetermined by their intended function. Once in the appropriate zone, these cells are termed "neurons," and their proliferation virtually ends by the day of birth (Miller, 1988). In addition, a programmed neuronal death begins at this stage, which is intended to end a

few days after birth, but which may be influenced by the lingering presence of ethanol (West *et al.*, 1990). (Figure 1) The final neuronal connections form as growth continues throughout the first two years of an infant's life (Bloom *et al.*, 1986). Hence, the vulnerability of brain neural development is heightened during weeks 3-16 but extended until birth, and its direct susceptibility to FAS effects lasts into the first two years of life (West *et al.*, 1990; Bloom *et al.*, 1986). However, there may be lingering effects on neuronal connections and growth that have not yet been researched.

Thus, three explanations for microcephaly should be studied: cell migration impairment, cell growth interference, and inhibition of mitotic activity as a direct result of prenatal ethanol exposure. Although each alone may be wholly responsible for microcephaly, it conceivably results from some combination of the three. In rats, ethanol has been shown to have profound effects on neuronal generation based upon disruptions in neuronal proliferation and migration (Miller, 1988). It was not clear from Miller's research, however, whether mitotic activity was a direct cause of microcephaly. Therefore, the present study focused on the long-term mitotic activity within the developing chick brain. To determine the effects of ethanol on this mitotic activity, a radioactive precursor was incorporated into the developing DNA prior to ethanol absorption.

Animal models have proven indispensable as a means to study alcohol-related developmental abnormalities because of the ease in studying large, very controlled populations in a shorter period of time without sacrificing human lives. The chick model is chosen for this research for three key reasons. First of all, the chick can be directly related to previous studies at

Washington and Lee concerning alcohol-affected brain development and FAS. Secondly, the correlation between human and chick brain development is high because the susceptible period begins and ends at approximately the same time for all vertebrates (Gorman, 1993). The chick is especially useful because its three week incubation period mirrors the developmental events within the trimesters of human pregnancy, allowing valid developmental comparisons. Finally, the chick's chorioallantoic membrane (CAM), which surrounds the embryo, is homologous to the human placenta. Thus, application of ethanol directly to the CAM layer mimics the ethanol exposure of the developing human exposed to ethanol-laden maternal blood in the placenta (Figure 2).

These experiments utilized incorporation of ^{3}H- thymidine into precipitable brain DNA as a measure of mitotic activity. Gorman (1993) found that infrequent, "binge" drinking has a more detrimental effect on brain development than prolonged, "chronic" abuse of alcohol. To simulate detrmimental "binge" levels, 5 μL of ethanol was in ected directly on the CAM layer on days 4, 10, and 16 (representative of one "binge" per trimester). Chicks which had received this treatment showed significantly suppressed brain and body weights when compared to untreated chicks and those which received 3 μL of ethanol on days 4, 6, 8, 10, 12, 14, and 16, simulating "chronic" drinking patterns (Gorman, 1993). Because these effects were so strong, this "binge" dosage and regimen were chosen for these experiments. In addition, [^{3}H]dT was added to the ethanol in order to study the amount of its incorporation into brain DNA. It was hypothesized that lower levels of [^{3}H]dT would be found in ethanol-treated chicks as compared to untreated or control groups because ethanol would have direct suppressive effects on mitotic activity.

This study replicated Gorman's results in both brain and body weight suppression as a direct result of high doses of ethanol exposure. However, it was not found that mitotic activity was the explanation for such events. No significant difference was found in the amount of [^{3}H]dT in ethanol treated or control groups, implying that ethanol does not directly affect mitotic activity of prenatal chick brain cells.

III. Materials and Methods

Eight dozen fertilized chicken eggs were obtained from Carolina Biological Supply Company. The eggs were randomly divided into two groups of 36 each and a third group of 24, and they were cared for according to the guidelines outlined in the publication "Carolina Fertile Chick Eggs Handling Instructions." The eggs were placed in an incubator heated to 38.5 C with a pan of water to provide it with approximately 80% humidity (Gorman, 1993).

[Methyl-^{3}H] dT was obtained from RBI with a concentration of 1.0 mCi/mL and a specific activity of 83.2 Ci/mmol.

The first set of eggs received 5 µl of 0.5% ethanol in saline solution with 0.012 µmol/ mL [Methyl-^{3}H] dT on days 4, 10, and 16. The second group served as "controls" and received 5 µl of a 0.75% saline solution with 0.012 µmol/ mL[^{3}H] dT on days 4, 10, and 16. The third group of 24 eggs served as "normals" and were left untreated for purposes of comparison.

Solutions were applied to the CAM using a Hamilton syringe inserted through a 0.8 cm square window cut into the eggshell directly above the embryo. The embryo was located by "candling" on day 4 of incubation (Southard, 1990). The surface of the egg above the embryo was sterilized using a swab of 70% EtOH and a dremmel tool with a cutting disc was used to cut a small slit in the blunt end of the eggshell, where the air sac is located, to allow the embryo to fall away from the surface prior to cutting the window. A square piece of scotch tape was placed over the hole to prevent contamination. The tape was then pierced with a Hamilton syringe for each subsequent dose.

On the nineteenth day, the chicks were removed from their shells and those that were alive, as indicated by proper development and muscle movement, were sacrificed by decapitation using dissecting scissors. This process was immediately followed by brain removal using microscissors and forceps to open the cranium, and then removing the brain by lifting it from its cavity using a microspatula. Both brain and body weights were measured using an analytical balance and recorded to the nearest microgram. The brains were then frozen at -20 C in separate scintillation vials until further use.

Both EtOH-treated and control groups were used for the whole-brain homogenate study. The DNA was precipitated from the homogenate using perchloric acid, and the radioactive precursor incorporation into DNA was determined as in Appendix A.

IV. Results

Four brains treated with ethanol and tritiated thymidine (EtOH + [^{3}H]dT) and four brains treated with tritiated thymidine alone ([^{3}H]dT controls) were available for the homogenate study. In addition, twelve brains which remained untreated were obtained on day 19 for comparison studies. All other embryos were dead or showed considerable pathology and were discarded.

The brains were weighed and recorded (Table 1). T he EtOH treated group mean was 0.387g, and for the control group the mean was 1.208g. The mean brain weight for the untreated group was 0.392g. A t-test was performed and showed a significant difference between the EtOH treated group and controls, $t(6) = -5.193$, $p < 0.001$, but revealed no other significant differences. (Table 5) These findings demonstrate that microcephaly occurred as a direct result of prenatal exposure to EtOH.

In order to determine whether smaller brain size in EtOH treated embryos was due to specific action in the brain or whether it was due to overall smaller whole body size, the ratio of brain to body weight was calculated (Table 2). Untreated brains had a mean ratio of 0.044, SD = 0.007. Surprisingly, the EtOH treated group had a slightly higher ratio ($\mu = 0.054$, SD = 0.015) than the control group ($\mu = 0.039$, SD = 0.010), but a t-test showed no significant difference between the two treatment groups. Thus, it appears that the ethanol-induced microcephaly found in this study reflects a generalized lower hatching weight.

Parameters for the measurement of mitotic activity were formed using three standard curves were established for Burton's DNA assay, with ranges from 0 to 10 µg DNA/mL 10% PCA, 0-100 µg DNA/mL 10% PCA, and 0-500 µg DNA/mL 10% PCA. These were all read at

5% of their original concentrations due to excess color formation. Absorbance was read at 595nm, the lambda max for the assay, and 700 nm to account for nonspecific material. The $A_{595-700}$ values for each experimental sample was evaluated against these standard curves so that nonspecific absorbance readings were eliminated. It was found that the 0 - 10 μg DNA / mL 10% PCA curve was most useful for these experiments. A linear regression analysis yielded a correlation coefficient of $r = 0.968$ (Figure 3).

The eight brains were homogenized, and a colorimetric assay was performed (Appendix A). Mean percent composition for the EtOH treated brains was 0.018%, and for the controls $\mu = 0.022\%$. A t-test was performed, and no significant difference was revealed ($t(6) = -1.018$, $p > 0.05$). This unexpected value demonstrates a failure for ethanol to directly affect mitotic activity in the developing brain.

Finally, a scintillation counting was performed on 100 μL of each brain homogenate sample to determine radioactivity of the DNA. Data were expressed as DPM / μg DNA (Table 4). The mean DPM / μg DNA for the EtOH treated brains was 20271.885 DPM / μg DNA, and for the controls the mean was 4876.54 DPM / μg DNA. Again, a t-test was performed but yielded no significant difference between the two treatment groups ($t(6) = 0.720$, $p > 0.05$). Thus, these results reinforce previous results showing the unexpected failure of direct ethanol-induced mitotic effects.

A summary of the results demonstrates definite ethanol-induced microcephaly correlated with decreased body weight. However, this microcephaly is not caused by a lack of mitotic activity, as evidenced by the lack of significant differences in DNA percent composition and

radioactivity between ethanol-treated and control groups. Instead, cell migration and overall growth may be more affected by ethanol treatment, or they may have cumulative effects which cannot be noted independently.

V. Discussion

Previous research has shown many debilitating physiological and mental effects of FAS, ranging from retardation to disfiguration (Moore, 1988). These studies have lead to investigations into FAS's exact mechanisms on the cellular level, especially in the developing brain. Based upon developmental research, it has been hypothesized in this study that microcephaly, one of the most common and notable effects of FAS, is likely to be caused by at least one of three factors: a decrease in mitotic activity, a failure of cells to grow once they are formed, or a failure of cells to migrate and differentiate.

These data support the conclusion that microcephaly in the chick is a direct result of high ethanol consumption (see Gorman, 1993). However, it was also shown that this microcephaly is accompanied by a decrease in body weight, supporting an effect of global growth deficiency as a direct result of high levels of prenatal alcohol exposure. The chick was again shown to be an excellent model for demonstrating these effects of FAS.

However, data from this investigation do not support the hypothesis that smaller brain weight and size are caused by a decrease in mitotic activity. Neither the percent composition DNA studies nor the scintillation assay showed any significant difference between groups treated with ethanol with [^{3}H]dT and control groups treated with [^{3}H]dT in saline solution.

Simultaneous research by Allen (1995) shows no significant differences in cell density or differentiation between ethanol treated and control brains. Thus, further investigation into the effects of excess ethanol consumption by pregnant mothers should include cell growth and migration studies in con unction with further studies of mitotic activity. If all three of these

factors could be compared simultaneously using the same brain material, then more exact comparisons could be made between treated and untreated brains, and cumulative effects which are not independently significant may be discovered.

In addition, more subjects are needed for statistically significant comparisons to be made. Extreme care must be taken in handling the developing chicks while injecting them with foreign substances, for infection is probably the leading cause of mortality among prehatching chicks. A gross mortality rate of 50% must be anticipated, as noted in the group of untreated chicks.

The chick brain was not found to be the best source for investigations into the exact causes of microcephaly for several reasons. These include: the high variability in "normal" chick brain size, weight, and density even when unmolested, the variability within treated brain weights and densities, and the high mortality rate and susceptibility to infection of the developing chick embryo. Other factors associated with FAS may be studied more easily in the chick, however. For example, few longitudinal studies have been performed investigating the effects of FAS on learning and coordination skills, which can easily be studied in the chick post-natally. The chick's abbreviated life span could be compared to the longer span of a human and effects would be recorded all the way into "adolescence" by teaching the chick a simple learning paradigm.

A means to study mitosis, cell growth, and migration should be simultaneously executed using light microscopy and autoradiography techniques for histology studies. It is also suggested that further possibilities of causation and mechanization be proposed in researching ethanol's effects on a cellular level. One key factor in studying brain development is the susceptible

growth period, which, in humans, lasts for several years. Because the neurons grow both in size and in the number of connections, and because the mental and growth retardation effects of FAS may not be noted for several years after birth, future studies need to involve post-hatching chicks and brains to fully understand ethanol's effects. For example, perhaps prenatal ethanol exposure has set up an environment in which the cells are unable to complete the formation of future connections. Such an effect would not be seen in the day 19 brains studied in this investigation.

Additional biochemical explanations for growth inhibition and other repressive effects must be investigated as well. Allen (1995) showed significant differences in cell density in the thalamus based upon ethanol treatment. Research into the relationship between this finding and lack of REM sleep, as well as problems with memory retention, should focus on neurotransmitter concentrations and receptors, since the thalamus is both a major relay center in the midbrain and has been linked to sleep and memory. Specifically, a relationship may exist between high levels of prenatal ethanol exposure and electrophysiological effects, as noted by Yool and Gruol (1987), which may be caused by changes in the amount of neurotransmitters that the neurons are able to produce or receive. Studies by Druse (1981, 1992) have shown considerable postnatal ethanol-induced effects on increased dopamine production in the frontal cortex, but specific research into the prenatal dopamine levels is lacking.

It is important, too, to note the inability to detect small differences in whole brain studies. Druse (1981) stressed the importance of regional neurotransmitter studies because of the relatively small levels which normally exist. An optimal study of FAS effects, then, would include regional investigations into mitotic, growth, and migration effects, as well as examining

the levels of dopamine or other relevant neurotransmitters in several regions of the brain. Although difficult, such a study would provide the missing information into possible cumulative effects of cell processes, as well as investigate further neurotransmitter effects. Comparisons could be made to determine where the most detrimental effects occur, as well as which of the proposed mechanisms has the most power in determining the final FAS outcome.

VI. Bibliography

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Appendix A: The Diphenylamine Method for the Estimation of DNA.

A sensitive but highly selective method of DNA estimation is necessary to determine small changes in content of fetal brain cells. The diphenylamine reaction of Burton (1956) as improved by Giles and Myers (1965) is used exclusively in these studies. Although the reaction has great specificity for 2-deoxyribose, sight ribose reactivity has been found; therefore, RNA is routinely removed from the samples prior to DNA determination. The diphenylamine reacts at low pH with 2-deoxyribose to yield a blue colored product whose absorbance at 595 nm is determined by spectrophotometry. Absorbance at 700 nm is used to determine non-specific light scattering. (Wielgus, 1977)

Solutions:

A. 1.6% (w/v) aqueous acetaldehyde (CH_3CHO; Mallinckrodt).

B. Diphenylamine reagent: 1.5g diphenylamine in 100 mL glacial acetic acid and 1.5 mL concentrated sulfuric acid. Just before needed, add 0.1 mL 1.6% acetaldehyde to 20 mL of reagent.

C. 10% (1.6N) PCA.

D. 3.2% (0.5N) PCA.

Procedure:

DNA Extraction

1) Homogenize weighed chick brain in 1 mL 0.5 N PCA / g tissue.
2) Wash tissue grinder in equal volume of 0.5 N PCA; pool with homogenate.
3) Extract DNA at 70 C for 20 minutes.
4) Cool on ice for 10 minutes.
5) Centrifuge at 4 C at 5000g for 20 minutes, discard supernatant.
6) Add equal volume 0.5N PCA, resuspend and centrifuge as above, discard supernatant.
7) Resuspend in equal volume 1.6 N (10%) PCA.

8) Aliquot 250 µL into scintillation vial for counting.
9) Aliquot 500 µL into a tube for DNA assay.

DNA Determination

1) To 500 µL DNA / 1.6 N PCA add 1.0 mL diphenylamine reagent, incubate at 30 C for 16 hours.
2) Determine absorbance against reagent blank at 595 nm and at 700 nm; calculate $A_{595-700}$.

Table 1 : Brain Weights in grams of Day 19 Chick Embryos. Treatments were either "None" (no injection given), "EtOH treated" (injected with 5 µl of EtOH and [^{3}H]dT on days 4, 10, and 16), or "Controls" (injected with 5 µl of Ringer's with [^{3}H]dT on days 4, 10, and 16). As seen from the mean brain weights, EtOH treated chicks had significantly smaller brain weights than controls. Thus, prenatal ethanol exposure is a direct cause of microcephaly.

Treatment

None (N = 12)	**EtOH treated (N = 4)**	**Controls (N = 4)**
µ = 0.392	**µ = 0.387**	**µ = 1.028**
SD = 0.042	**SD = 0.178**	**SD = 0.171**

Table 2 : Ratios of brain weight / body weight of Day 19 Chick Embryos. Treatments were either "None" (no injection given), "EtOH treated" (injected with 5 µl of EtOH and [^{3}H]dT on days 4, 10, and 16), or "Controls" (injected with 5 µl of Ringer's with [^{3}H]dT on days 4, 10, and 16). Although brain weight has been shown to decrease as a result of prenatal ethanol exposure, the total body weight decreases as well, leading to a much smaller difference in the brain weight / body weight ratio of EtOH treated and control groups.

Treatment

None	**EtOH treated**	**Controls**
μ = 0.044	**μ = 0.054**	**μ = 0.039**
SD = 0.007	**SD = 0.015**	**SD = 0.010**

Table 3 : Percent Composition of DNA / Brain Weight. Treatments were either "None" (no injection given), "EtOH treated" (injected with 5 µl of EtOH and [^{3}H]dT on days 4, 10, and 16), or "Controls" (injected with 5 µl of Ringer's with [^{3}H]dT on days 4, 10, and 16). Although ethanol treatment yielded a slightly lower percent composition of DNA, there is no significant difference between EtOH treated and control specimens.

Treatment

None	EtOH treated	Controls
$\mu = 0.024$	$\mu = 0.018$	$\mu = 0.022$
SD = 0.005	SD = 0.006	SD = 0.006

Table 4 : Scintillation counts of DPM per μg DNA, as calculated from known percent DNA composition for each sample. Treatments were either "EtOH treated" (injected with 5 μl of EtOH and [^{3}H]dT on days 4, 10, and 16) or "Controls" (injected with 5 μl of Ringer's with [^{3}H]dT on days 4, 10, and 16). Because [^{3}H]dT would be taken up preceding EtOH incorporation into newly replicated DNA, a significant decrease in radioactivity of the brain homogenate, counted in DPM / μg DNA, would result if ethanol was directly inhibiting mitosis. However, no such effect was found.

Treatment

EtOH treated	**Controls**
μ = 20271.885	**μ = 15395.34**
SD = 8694.928	**SD = 10396.902**

Table 5 : Mean values and differences found between brains. Treatments were either "EtOH treated" (injected with 5 µl of EtOH and [^{3}H]dT on days 4, 10, and 16) or "Controls" (injected with 5 µl of Ringer's with [^{3}H]dT on days 4, 10, and 16). As noted from the significance of the t values, prenatal ethanol exposure caused a significant decrease in brain weight only. Because there was no significant difference between the ratios of brain weight / body weight, percent DNA composition, or DPM / µg DNA, it is shown that ethanol does not have a direct impact in decreasing mitotic activity in the developing brain.

Comparison	**EtOH treated**	**Controls**	**Difference**	**t Value**
Brain Weight	$\mu = 0.387$g	$\mu = 1.028$g	d = 0.641g	$t(6) = -5.193$ $p < 0.001$
Brain Weight/ Body Weight	$\mu = 0.054$	$\mu = 0.039$	d = 0.015	$t(6) = 1.623$ p = NS
Percent DNA Composition	$\mu = 0.018\%$	$\mu = 0.022\%$	d = 0.004%	$t(6) = -1.018$ p = NS
DPM / µg DNA	$\mu = 20271.885$ DPM/µg DNA	$\mu = 15395.34$ DPM/µg DNA	d = 4876.54 DPM/µg DNA	$t(6) = 0.720$ p = NS

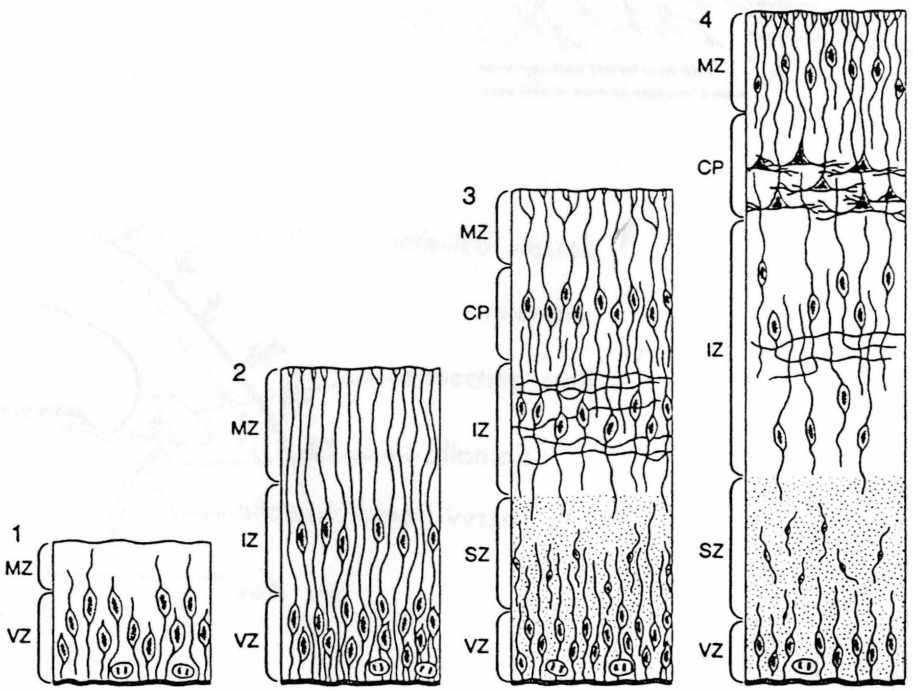

Figure 1 : The wall of the developing brain at the earliest stage (1) consists of only a "pseudostratified" epithelium, in which the ventricular zone (VZ) contains the cell bodies and the marginal zone (MZ) contains only the extended outer cell processes. When some of the cells lose their capacity for synthesizing DNA and withdraw from the mitotic cycle (2), they form the intermediate zone (IZ). In the forebrain the cells that pass through this zone aggregate to form the cortical plate (CP), the region in which the various layers of the cerebral cortex develop (3). At the latest stage (4), the subventricular zone (SZ) forms as a second proliferative region in which many glial cells and some neurons are generated. (Cowan, 1979)

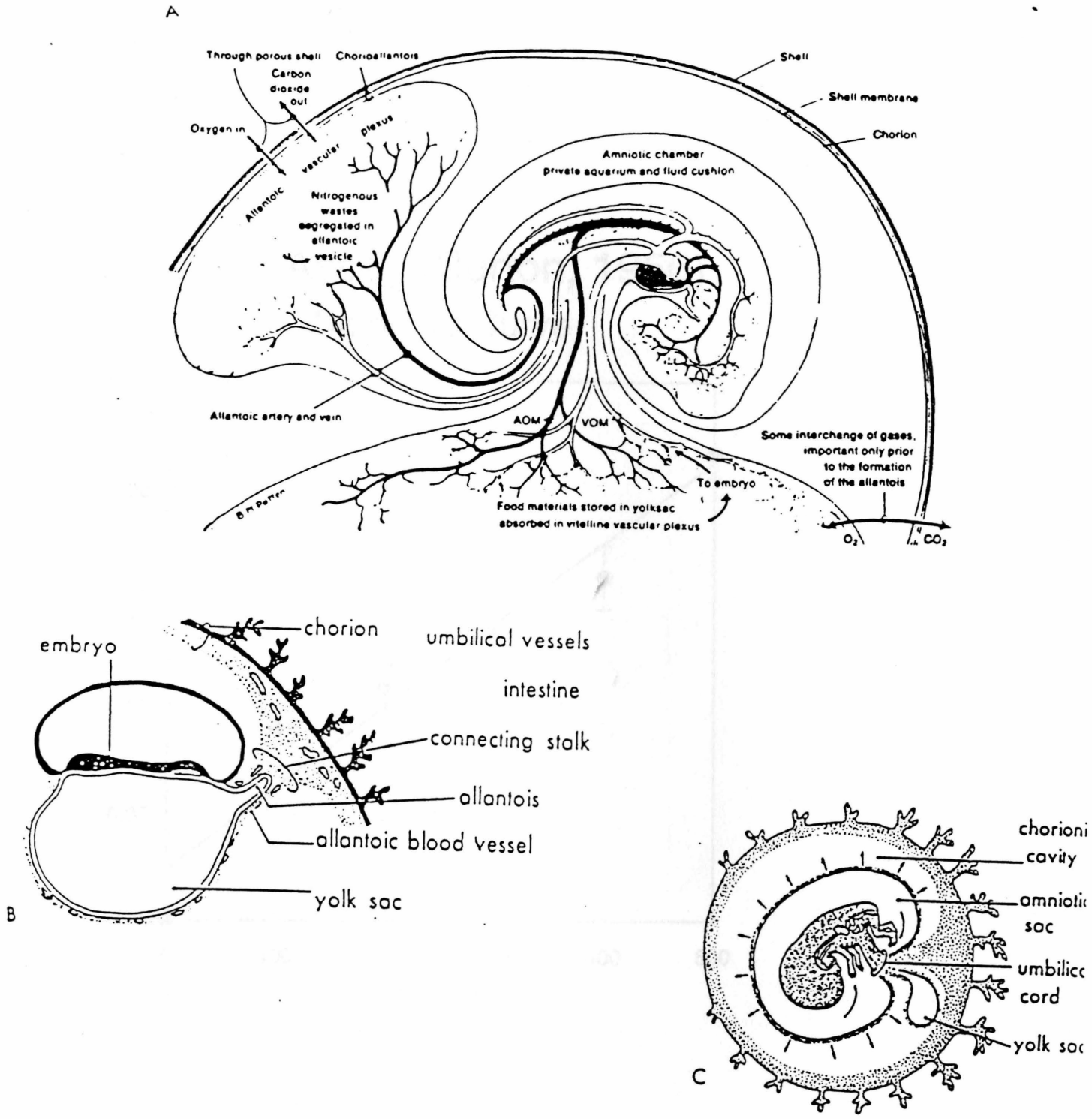

Figure 2 : Diagrams illustrating the homology between the chick chorioallantoic membrane and the human placenta. A) 4-day chick embryo; B) Three week human embryo; C) Ten week human embryo. Abbreviations: AOM, omphalomesenteric (vitelline) artery; VOM, omphalomesenteric (vitelline) vein. (Matthews, 1987)

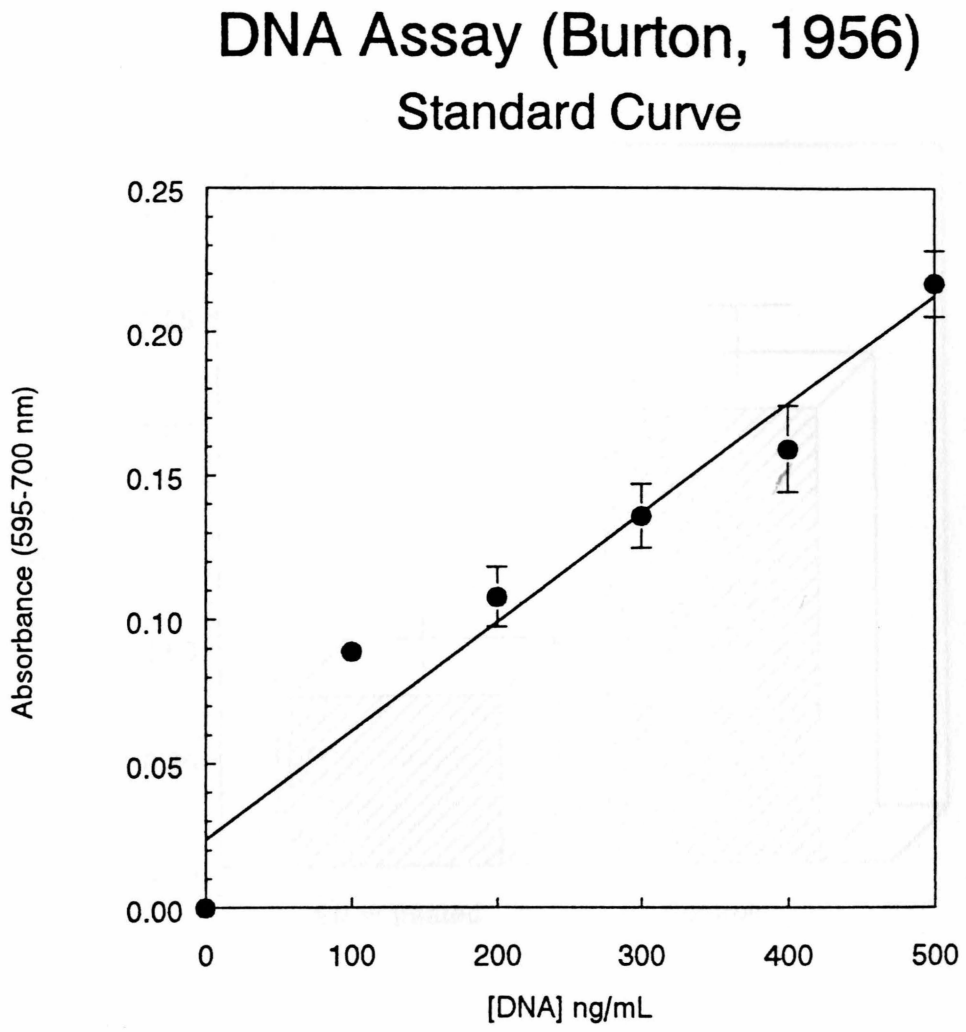

Figure 3 : Standard curve established for DNA determination by the diphenylamine method. (Burton, 1956)

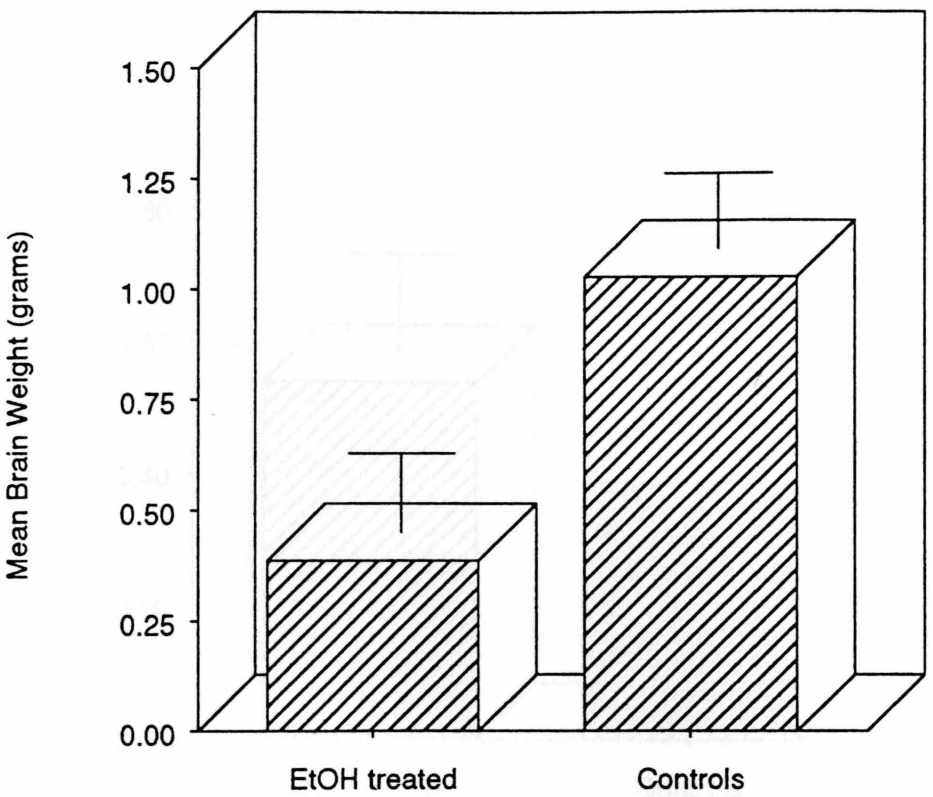

Figure 4 : Comparison of mean brain weight based upon treatment regimen. Treatments were either "EtOH treated" (injected with 5 µl of EtOH and [^{3}H]dT on days 4, 10, and 16) or "Controls" (injected with 5 µl of Ringer's with [^{3}H]dT on days 4, 10, and 16). A significant difference was found between the means: $d = 0.641$g, $t = -5.193$ ($p < 0.001$).

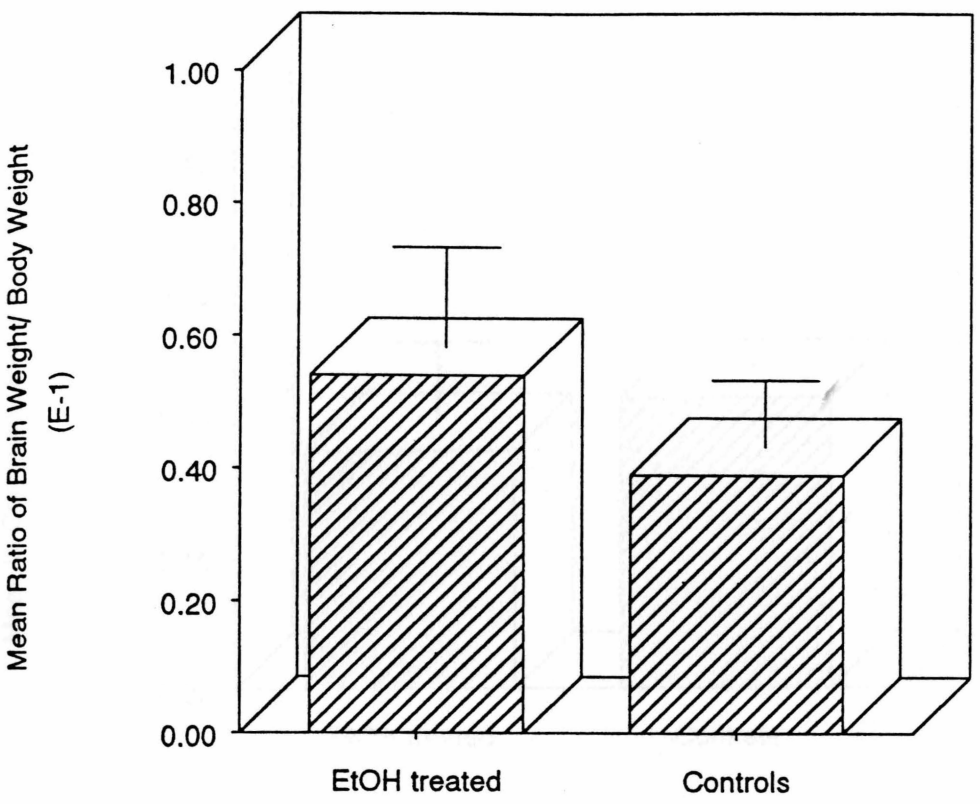

Figure 5 : Graphical representation of difference between mean ratios of brain weight/ body weight. Treatments were either "EtOH treated" (injected with 5 µl of EtOH and [^{3}H]dT on days 4, 10, and 16) or "Controls" (injected with 5 µl of Ringer's with [^{3}H]dT on days 4, 10, and 16). No significant difference was found between the means based upon treatment regimen (d = 0.015).

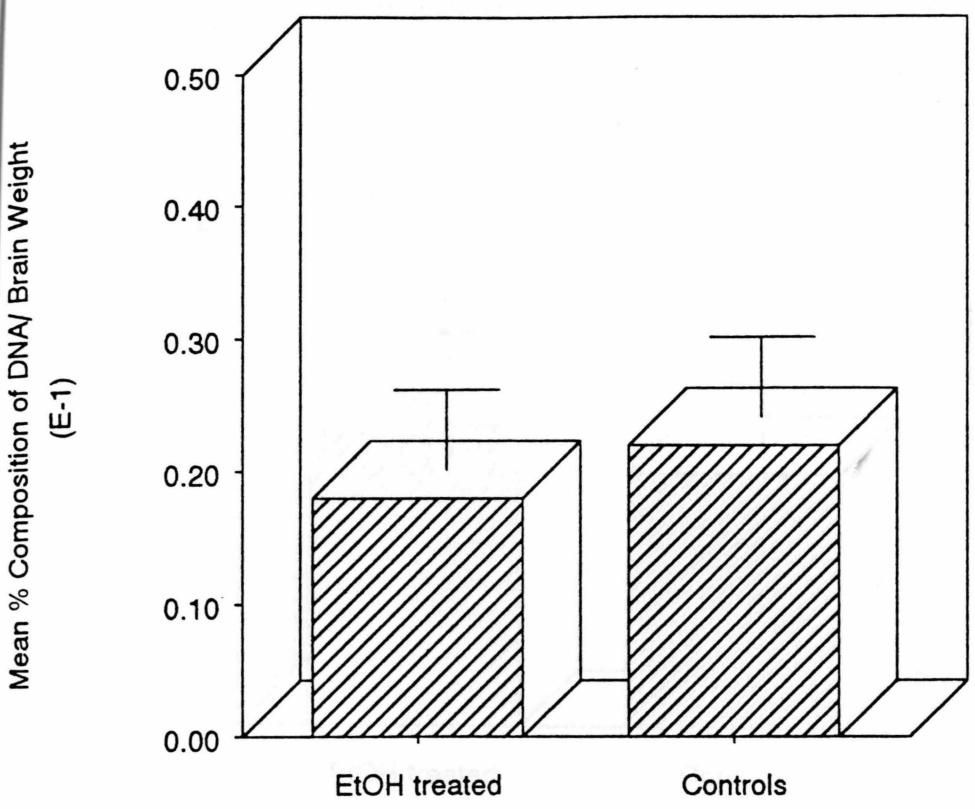

Figure 6 : Difference between mean percent composition of DNA per brain weight. Treatments were either "EtOH treated" (injected with 5 µl of EtOH and [^{3}H]dT on days 4, 10, and 16) or "Controls" (injected with 5 µl of Ringer's with [^{3}H]dT on days 4, 10, and 16). No significant difference was found based upon treatment regimen (d = 0.004%).

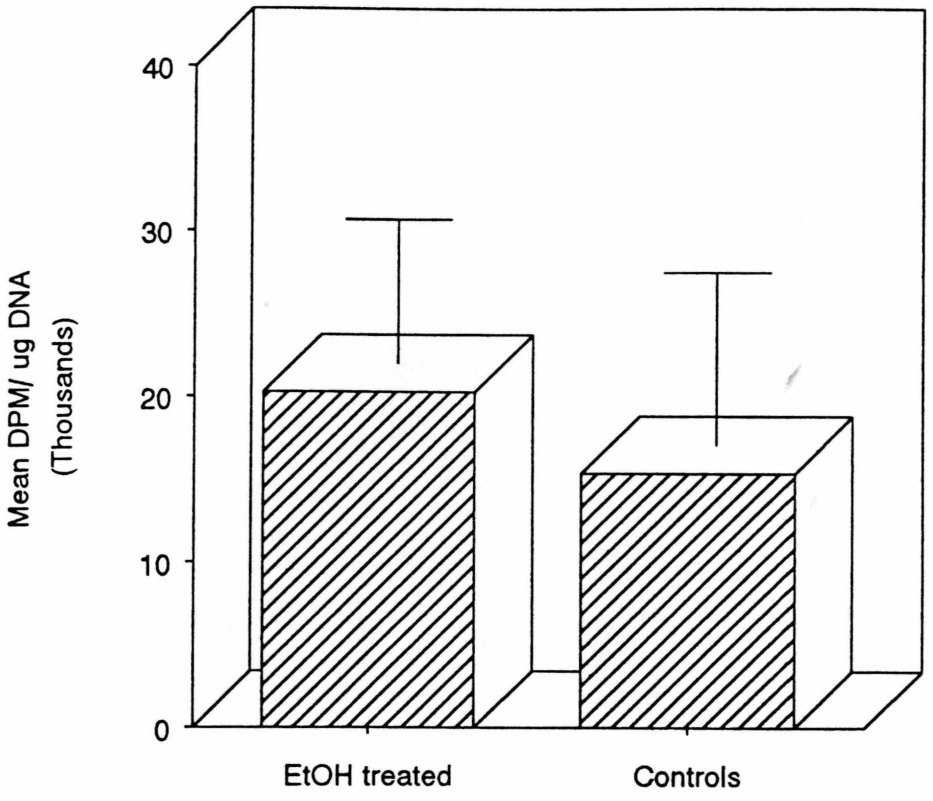

Figure 7 : Comparison of mean DPM/ μg DNA. Treatments were either "EtOH treated" (injected with 5 μl of EtOH and [^{3}H]dT on days 4, 10, and 16) or "Controls" (injected with 5 μl of Ringer's with [^{3}H]dT on days 4, 10, and 16). No significant difference was found based upon treatment regimen (d = 4876.54 DPM/ μg DNA).