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Report

Teratogenic and Behavioral Anomalies Induced by Acute Exposure of Mice to Ethanol and Their Possible Relation to Fetal Brain DNA Synthesis

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Physical and behavioral anomalies of fetal alcohol syndrome were studied after the i.p. administration of a single 3- or 6-g/kg dose of ethanol (25%, v/v) to gravid mice on either day 15 or day 18 of gestation. The physical effects of ethanol administered on either day 8, day 10, or day 12 of gestation (N = 6/group) were also examined and compared to the saline-administered controls. The identification of these anomalies and the effect of ethanol on the rate of fetal brain DNA synthesis were investigated. The physical anomalies were identified by standard procedures. Behavioral anomalies were measured as the inhibition of the development of various neonatal reflexes (N = 6–13/group) as compared to the saline-administered controls. The possible mechanism for these ethanol-induced abnormalities was identified by using [^{3}H]thymidine to measure the rate of DNA synthesis (N = 6/group) in fetal mouse brains. Blood alcohol concentrations (N = 6/group) ranged from 410.2 mg/dl at 30 min to 25.8 mg/dl at 4.5 hr following the dosage of 3 g/kg of ethanol. Concentrations following the dosage of 6 g/kg of ethanol ranged from 753.7 mg/dl at 15 min to 127.1 mg/dl at 10.5 hr postinjection. Fetal and maternal weight gains were significantly inhibited compared to those of the controls. Various cranial facial, urogenital, skeletal, and cardiovascular anomalies were observed ($P \leq 0.05$). Delays in the onset of the air and surface righting, visual placing, and negative geotaxis reflexes were observed for the ethanol-treated neonates, as compared to control values. The rate of DNA synthesis in the ethanol-group fetal mouse brains was significantly less than the control values.

Key Words: teratogenic and behavioral anomalies; fetal alcohol syndrome; brain DNA synthesis.

INTRODUCTION

Concern with the deleterious effects of alcohol upon the developing conceptus has been recorded for centuries. A published report by Jones and Smith (1) was one of the first to observe a direct correlation between the maternal use of alcohol and birth defects. These authors used the term "fetal alcohol syndrome" (FAS) to describe a triad of symptoms which include mental impairment, craniofacial malformations, and intrauterine and postnatal growth reduction.

The insidious effects of ethanol upon mental development has led many investigators to use animal models in an effort to mimic this deleterious condition. Although there have been numerous investigations into the effects of ethanol upon the behavioral reflexes of animals, not all of the results are in agreement. In three independent studies, one study reported that prenatally administered ethanol caused a delay in the appearance of the surface righting reflex (2), another study observed no apparent effect (3), and the third investigation observed an increased appearance (4) of this reflex as compared to the control values. A similar disparity of data is seen in testing for the visual placing reflex: two studies (2,4) observed no change in this neonatal reflex after *in utero* ethanol exposure and a third study (5) reported an inhibition in the development of this reflex as compared to controls.

Far fewer investigations have attempted to determine the mechanism by which ethanol causes its specific neurological effects and even these studies have reported conflicting results. Previous studies have shown that *in utero* ethanol exposure can cause an inhibition of brain myelin production (6–8) and leucine incorporation (9). Other studies have investigated the rate of brain RNA synthesis and have observed ethanol to cause a decrease (9,10), no significant change (11), or an increase in the rate of brain RNA synthesis (12). The amount of protein synthesis in the neonatal brain tissue has also been investigated and was reported to be decreased (13,14) or not affected (11) following maternal ethanol dosage. A parameter that would appear to be most critical in the determination of a normal-functioning central nervous system would be the content and rate of synthesis of DNA. Any deficits in the replication of DNA would have dramatic results upon the mental capacity of the animal. Several studies have attempted to determine the effect of ethanol on the rate of DNA synthesis in fetal brains and have reported a decrease (9,15) or no change (11,16) as compared to controls.

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This investigation was designed to determine the effects of a single dosage of ethanol on the cellular mitotic activity of the fetal brain and the subsequent development of fetal abnormalities and neonatal reflexes.

EXPERIMENTAL

Animals

CF-1 albino (Charles River Breeding Laboratories, Wilmington, Mass.) mice weighing approximately 25 g were used in all of the following procedures. All female mice were caged in aggregates of 10 each and were acclimated for approximately 2 weeks. Following the acclimation period, those females were allowed to mate. The male mice were housed in individual cages measuring 12.5 × 15 × 10 cm, each with a wire-mesh front and floor (Norwich Wire Works, Norwich, N.Y.). Food (Purina Laboratory Chow, Ralston-Purina Co., St. Louis, Mo.) and tap water were offered *ad libitum;* artificial light was supplied on a 12-hr light/dark cycle. The temperature was maintained at between 22 and 26°C.

Drug and Solutions

Normal saline (sodium chloride 0.9% solution, Abbott Laboratories, Chicago, Lot 63-086-JT exp. 4/86) was used as the vehicle for the ethanol (ethyl alcohol, 95%; Pharmaco, Publicker Chemical Co., Philadelphia) solutions (25%, v/v); solutions were prepared fresh weekly and stored refrigerated. Normal saline was administered to all dams in the vehicle control group. All injections were made intraperitoneally, using either a 1-cm^3 (B-D tuberculin syringe, 0.5-in., 27-gauge needle; Becton, Dickinson and Co., Lot 2L310, Rutherford, N.J.) or a 3-cm^3 (B-D syringe, 0.5-in., 26-gauge needle; Becton, Dickinson and Co., Lot 5570, Rutherford, N.J.) syringe.

Breeding, Group Selection, and Treatment

The breeding procedure has been described previously (17). Gravid females were randomly assigned to one of the three sections of the study: teratology, neonatal behavioral reflexes, and DNA synthesis analyses. The teratology section of the study consisted of 10 groups, with each group containing six animals. The dams were either untreated or administered saline or 3 or 6 g of ethanol/kg of body weight. Each dam was dosed only once, on a single day of gestation (day 8, 10, 12, or 15 of gestation). The neonatal behavioral reflexes section of the study consisted of 40 groups,[3] with each group containing between 6 and 13 animals; these dams were either untreated or administered a single dose of saline or 3 or 6 g/kg of ethanol on either day 15 or day 18 of gestation. The neonates from each of these dosage groups were tested for only one of the following neonatal behavioral reflexes: negative geotaxis, surface righting, auditory startle, air righting, and visual placing. The DNA synthesis analyses section of the study consisted of 12 groups, with each group containing six animals; these dams were either untreated or administered a single dose of 3 or 6 g/kg of ethanol on either day 15 or day 18 of gestation.

Blood Alcohol Analyses

Non-gravid CF-1 female mice were administered a single dosage of 3 or 6 g/kg of ethanol, and at the appropriate time intervals whole blood samples were obtained from the retroorbital plexus and analyzed for blood alcohol concentration (BAC). Samples were drawn in heparinized capillary tubes (Yankee, Micro-hemocrit, heparinized tubes, Lot 10610, Clay Adams, Becton, Dickinson and Co., Parisippany, N.J.) and analyzed by head-space analysis (19) using a gas chromatograph (Glowall 310 gas chromatograph, Glowall Corp., Willow Grove, Pa.; 5% Carbowax 20M on 60/80 Carbopack B column, Cat. No. 1-1766, Supelco Inc., Lot H02276). Concentrations were determined by using *n*-propanol (certified Fischer Scientific Co., Lot 731961, Chemical Manufacturing Division, Fair Lawn, N.J.) as an internal standard and comparing to standard curves.

Examination of the Teratology Fetuses

On day 18 (1 day prior to full term), each dam was weighed and sacrificed by cervical dislocation. The number and position of fetuses and resorption sites were recorded. Fetuses were weighed and checked for viability, sex, and gross external alterations. One-half of the fetuses were then randomly selected for staining prior to skeletal examination by the methods of Staples and Schnell (20). The remaining fetuses were prepared in Bouin's solution for soft tissue examination by the method of Wilson (21). The statistical significance of the observations was determined using Student's *t* test (22) and the uncorrected chi-square test for a binomial population (23).

Testing of Neonatal Behavioral Reflexes

The dams selected for behavioral testing were allowed to give birth in individual plastic cages with nesting material (Beta-chip, Hardwood Laboratory Bedding, Northeastern Product Corp., Warrensburg, N.Y). At the appropriate intervals the developing neonates were evaluated for the presence of one of the following behavioral reflexes: negative geotaxis, surface righting, auditory startle, air righting, and visual placing. For each of the tested reflexes, a litter was considered to possess a reflex only if each neonate in the litter performed the test satisfactorily in three of three trials. These tests were performed at approximately the same time each day and the results of the dosage groups were compared using Student's *t* test.

Analyses of the Rate of DNA Synthesis

The dams selected for this portion of the study were untreated or administered a single dose of 3 or 6 g/kg of ethanol 2 hr prior to the administration of 1 μCi/g of body weight [^{3}H]thymidine (5 Ci/mmol) ([*methyl*-^{3}H]thymidine, Amersham Corp., Arlington Heights, Ill., Cat. No. TRA120, batches 212 and 214) on either day 15 or day 18 of gestation. The dams were then sacrificed after the thymidine was al-

[3] Untreated control data were generated in conjunction with Mahalik and Gautieri and have been reported previously (18).

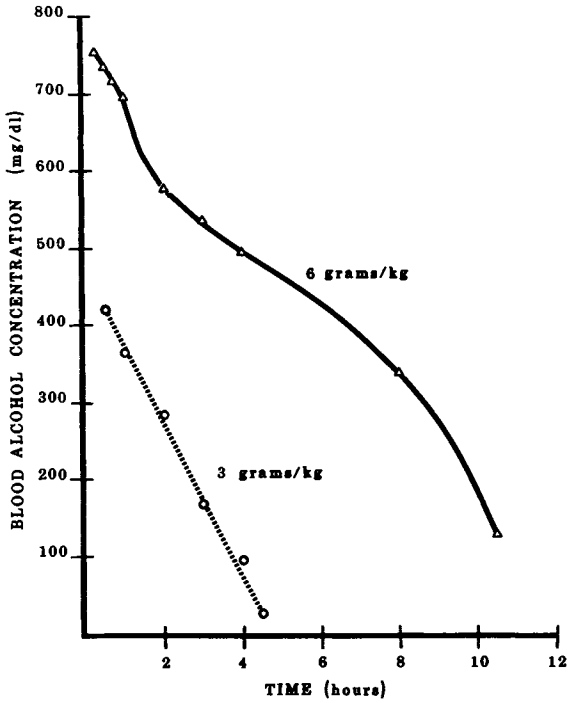

Fig. 1. Blood alcohol concentrations in CF-1 mice following ethanol administration. Nongravid CF-1 mice were administered a single dosage of either 3 or 6 g/kg of ethanol. Each point on the curve corresponds to six sampled animals.

lowed to incorporate for 1 hr. The fetuses were delivered by cesarean section; fetal brain tissue was extracted and pooled by litter. The analyses of the rate of DNA synthesis were done according to the procedure described by Dreosti *et al.* (15). The results of the dosage groups were compared using Student's *t* test.

RESULTS AND DISCUSSION

The blood alcohol concentration (BAC) ascertained during this investigation from a single dosage of 3 g/kg of ethanol ranged from 410.2 mg/dl measured at 0.5 hr postinjection of 25.8 mg/dl at 4.5 hr postinjection. The animals administered a single dosage of 6 g/kg of ethanol produced a higher BAC that ranged from 753.7 mg/dl measured at 0.25 hr postinjection to 127.1 mg/dl at 10.5 hr postinjection. The concentrations and times that ethanol was able to be detected in the maternal blood are comparable to those observed in other investigations (24–26). The concentration versus time curve presented in Fig. 1 is also similar to that of previously published studies (27). The metabolic degradation rates of ethanol in humans and mice are generally assumed to be approximately 100 and 550 mg/kg/hr, respectively. The significant difference in the metabolic rates of the two species allows some insight into the drug levels that must be attained to achieve an effect for both species. Animal studies have indicated that these rates are not altered by pregnancy (28).

Teratology

The data reported in this study indicate that ethanol administered during early, middle, or late gestation adversely affected the developing conceptuses. In the comparison of the 10 maternal/fetal characteristics with the saline controls, it appears that day 10 of gestation is the most vulnerable to teratogenic insult. For the dosage group administered 6 g/kg of ethanol on day 10 of gestation, 6 of 10 parameters compared to the saline control were significantly ($P \leq 0.05$) altered.

Ethanol administered on day 10, 12, or 15 of gestation resulted in dosage-dependent and/or significant ($P \leq 0.05$) reductions in maternal body weight gain and mean fetal weight as compared to saline control values. The number of

Table I. Mean Values for the Teratology Litter Data[a]

Treatment	Maternal weight gain (g)[b]	Fetal ratio, R/L	Resorption ratio, R/L	Mean fetal weight (g)	Sex ratio, R/L	Soft tissue abnormalities	Skeletal abnormalities
Untreated	27.3	6.7/5.8	0.3/0.0	1.25	6.3/6.2	0.0	6.7
Day 8							
Saline	24.7	5.0/5.5	0.0/0.6	1.18	5.3/5.2	0.6	6.8
3 g/kg ethanol	22.9	5.0/4.0	0.1/0.6	1.23	4.5/4.5	0.6	5.7
6 g/kg ethanol	23.3	4.7/4.8	1.0/0.5	1.05	5.2/4.3	5.0	13.3*
Day 10							
Saline	27.6	6.2/5.8	0.3/0.1	1.24	7.0/5.0	0.3	6.5
3 g/kg ethanol	27.4	7.2/4.7	1.0/0.5	1.22	6.0/5.8	0.6	7.0
6 g/kg ethanol	20.9*	4.8*/3.0*	1.3/1.8	1.06*	4.2*/3.7	1.8*	10.3
Day 12							
Saline	27.7	5.7/6.2	0.5/0.0	1.28	5.8/6.0	0.5	3.8
3 g/kg ethanol	21.9	5.2/4.3	0.8/0.1	1.21	5.2/4.3	1.8*	7.2
6 g/kg ethanol	21.3*	4.3/5.7	1.0/0.6	1.04*	5.3/4.7	2.2*	11.5*
Day 15							
Saline	26.9	5.7/6.3	0.1/0.8	1.18	6.5/5.5	0.1	6.2
3 g/kg ethanol	27.3	6.2/5.7	0.1/0.8	1.22	5.7/6.2	1.7*	6.8
6 g/kg ethanol	20.1*	5.0/4.3*	0.6/1.0	1.00*	5.0/4.3	1.3	10.3

[a] R/L, right and left uterine horns. All weights recorded in grams.
[b] Day 0 through day 18 of gestation.
* $p < 0.05$ compared to the saline control.

Table II. Significant Occurrences* of Fetal Abnormalities Induced by Ethanol Dosage[a]

Abnormality	Gestational day of dosage			
	Day 8	Day 10	Day 12	Day 15
Exencephaly	High	—	—	—
Maxillary hypoplasia	High	—	—	—
Hydronephrosis	—	High	—	—
Interventricular septal defect	—	—	High	—
Delayed ossification of the paws	—	—	High	—
Delayed ossification of the supraoccipital bone	—	High	High	High
Malformed sternebrae	Low; high	Low	High; low	—

[a] The low dosage of ethanol was 3 g/kg; the high dosage of ethanol was 6 g/kg.

* Significantly different from saline control values compared on a litter basis at $P \leq 0.05$.

resorptions for the ethanol dosage groups was not significantly ($P > 0.05$) different from the saline control values. However, a slight dosage-dependent increase in the number of resorptions may exist as a result of the administration of ethanol on day 10 of gestation. A significant ($P \leq 0.05$) reduction in the fetal ratio occurred for dams administered 6 g/kg of ethanol on day 10 or 15 of gestation as compared to the saline control values. The interpretation of these data is unclear as a dosage dependency for both left and right uterine horns was not observed. The number of males for the day 10 dosage group administered 6 g/kg of ethanol was significantly reduced as compared to the saline control values. The sex ratios for the remaining dosage groups did not appear to be significantly different ($P > 0.05$) and dosage dependent as compared to the saline control values. The mean number of soft tissue abnormalities was dosage dependent and/or significantly ($P \leq 0.05$) increased for litters administered ethanol on day 10, 12, or 15 of gestation as compared to the saline control values. These data indicate that ethanol has a greater potential to induce soft tissue abnormalities during the middle and late gestational periods. The mean number of skeletal abnormalities was dosage dependent and/or significantly increased for litters administered ethanol on day 8, 10, 12, or 15 of gestation as compared to the saline control values. These data indicate that ethanol administration can potentially induce skeletal abnormalities throughout the entire gestational period.

The significant fetal abnormalities that occurred as a result of ethanol administration are listed in Table II. Specifically, significant increases ($P \leq 0.05$) in craniofacial, urogenital, cardiovascular, and/or skeletal fetal abnormalities were observed in litters administered a single dosage of 6 g/kg of ethanol on day 8, 10, 12, or 15 of gestation as compared to saline control values. Significant increases ($P \leq 0.05$) in the number of skeletal abnormalities occurred for litters administered 3 g/kg of ethanol on day 8, 10, or 12 of gestation as compared to the saline control values.

The incidences of exencephaly and maxillary hypoplasia (Fig. 2) were significant ($P \leq 0.05$) for litters administered a single 6-g/kg dosage of ethanol on day 8 of gestation as compared to the saline control values. These craniofacial

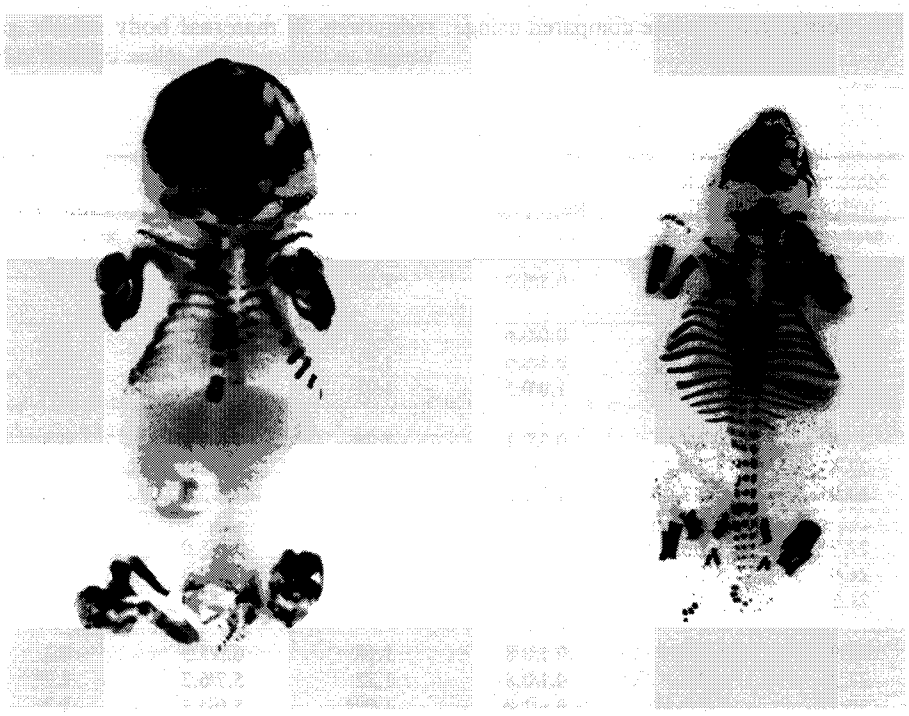

Fig. 2. Craniofacial and skeletal abnormalities induced by prenatal ethanol exposure. The day 18 fetal mouse specimen on the left is a control animal and the specimen on the right was treated prenatally with 6 g/kg of ethanol. The ethanol specimen has exencephaly, sternebra alterations, and mandibular and maxillary hypoplasia.

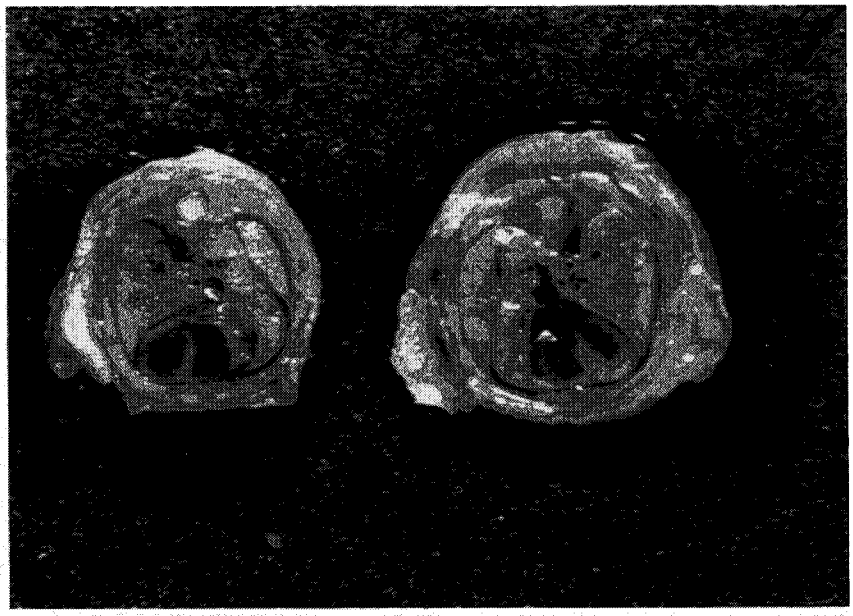

Fig. 3. Interventricular septal defect induced by prenatal ethanol exposure. Specimens are cross sections of gestational day 18 mice fetuses that have been treated with Bouin's solution. The section on the left is a control specimen and the one on the right was administered 6 g/kg of ethanol *in utero* and contains an interventricular septal defect.

malformations have been induced in previous studies (24,25,29) by an acute dosage of ethanol also administered on day 8 of gestation. The production of these malformations is probably a result of interference with normal somite formation and migration that is occurring during early gestation. A dosage-dependent and significant ($P \leq 0.05$) increase in the incidence of hydronephrosis was observed for litters administered a single 6-g/kg dose of ethanol on day 10 of gestation. Hydronephrosis has also been induced by previous investigators (29) who administered ethanol to dams on day 10 of gestation. Although this abnormality does not represent any gross defect of the kidney, it appears that ethanol has altered urinary flow and interrupted the proper development of the urinary system. The incidence of cardiovascular abnormalities (interventricular septal defect; see Fig. 3) was dosage dependent and significantly ($P \leq 0.05$) increased for litters administered 6 g/kg of ethanol on day 12 of gestation as compared to the saline controls. It may be possible that ethanol administered on day 12 of gestation may interrupt the endocardial cell masses from proper proliferation or cause retardation of the membraneous interventricular septum growth. Significant ($P \leq 0.05$) increases in skeletal alterations (malformed sternebrae, delayed ossification of the paws, and supraoccipital bone) were observed for litters administered 6 g/kg of ethanol on day 8, 10, 12, or 15 of gestation. Malformed sternebrae were also observed for litters administered 3 g/kg on day 8, 10, or 12 of gestation. These alterations may be due to a distortion of the correct cellular migration patterns and may cause abnormal bone ossification.

Other fetal abnormalities observed in this study that did not occur in significant ($P > 0.05$) numbers on a litter basis as compared to the saline controls included open eyes, cleft palate, ectopic ovary, and extra ribs.

Neonatal Behavioral Reflexes (Table III)

Although the neonatal reflexes that were tested in this study are innate and probably do not result from any type of learning process, they are polysynaptic and involve numerous portions of the brain. The development of these areas of the brain occurs throughout gestation and continues

Table III. Mean Values for the Day of Appearance of Neonatal Reflexes and Gestational Day of Ethanol Dosage

Reflex tested	Untreated control	Day 15			Day 18		
		Saline	3 g/kg	6 g/kg	Saline	3 g/kg	6 g/kg
Negative geotaxis	9.8	9.8	13.0*	12.8*	9.5	10.5	9.6
Surface righting	11.2	11.7	14.9*	17.3**	10.9	16.1**	18.6**
Auditory startle	15.5	—	15.0	16.8	—	15.2	15.9
Air righting	19.6	20.3	24.1**	25.4**	20.0	23.0**	25.4**
Visual placing	20.2	19.8	21.9*	23.5**	19.5	21.3**	22.3**

* Significantly different from saline control values at $P \leq 0.05$.
** Significantly different from saline control values at $P \leq 0.01$.

Table IV. Effect of Maternal Dosage of Ethanol on the Incorporation of [^{3}H]Thymidine into Fetal Brain DNA

Dosage group	N^a	Mean sp act of DNA (cpm/μg DNA)^b
Day 15 of gestation		
Control	6	77.6 ± 11.8
3 g/kg ethanol	6	47.8 ± 3.4*
6 g/kg ethanol	6	32.7 ± 5.2**
Day 18 of gestation		
Control	6	71/2 ± 17.3
3 g/kg ethanol	6	25.8 ± 3.6*
6 g/kg ethanol	6	24.5 ± 7.2*

[a] Number of animals per group.
[b] Standard error reported.
* Significantly different from control values at $P \leq 0.05$.
** Significantly different from control values at $P \leq 0.01$.

postnatally. Numerous portions of the mouse brain, including the cerebral cortex (30), corpus striatum (31), hippocampus pyramidal cells (32), and medial septal nuclei (33), have been identified as actively producing neurons on day 15 of gestation. Areas of the mouse brain generating neurons on day 18 of gestation are the cerebellum (34), dentate gyrus (32), and corpus striatum (31). Neuronal cells are more vulnerable to insult during their initial rapid generation period, and ethanol administered to the dams on either day 15 or day 18 of gestation may have dramatic effects upon these portions of the brain.

The offspring of the maternal animals dosed with ethanol on day 15 of gestation showed dosage-dependent and/or significant ($P \leq 0.05$–0.01) delays in the development of the negative geotaxis, surface righting, air righting, and visual placing reflexes, i.e., the age of the neonates from the ethanol-treated dams was significantly greater than the age of the neonates from the saline control dams to perform these reflexes successfully. The performance of the auditory startle reflex was not observed to be significantly ($P > 0.05$) different among the groups. A delay in the development of the air and surface righting and the visual placing reflexes of the neonates whose dams were treated with ethanol on day 18 of gestation was dosage dependent and significantly ($P \leq 0.05$) different as compared with the saline control values. The auditory startle and negative geotaxis reflexes did not appear to be affected by ethanol dosage to the dams on day 18 of gestation upon comparison to the control groups.

The rate of DNA synthesis was measured in fetal mouse brains following the administration of ethanol to the maternal animals. Using tritiated thymidine, the number of cells undergoing active DNA replication was approximated. The presence of a nucleoside salvage pathway allows for thymidine to serve as a means for the estimation of cell mitosis (35). The knowledge of which particular cell type is undergoing mitosis is critical to the predictions of the results of the damage induced. Since the dosage times used in this study are concurrent with neuronal cell mitosis, the reduction in the rate of DNA synthesis is a reflection of the neuronal cell population (36,37).

The rate of incorporation of labeled thymidine was dosage dependent and significantly ($P \leq 0.05$–0.01) inhibited for all dosage groups administered ethanol (3 or 6 g/kg) on either day 15 or day 18 of gestation as compared to the control values.

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