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Strain Differences in Developmental Vulnerability to Alcohol Exposure Via Embryo Culture in Mice

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Abstract

Background—Prenatal alcohol exposure can result in varying degrees of neurodevelopmental deficits, growth retardation, and facial dysmorphology. Variation in these adverse outcomes not only depends on the dose and pattern of alcohol exposure but also on less well understood interactions among environmental, genetic, and maternal factors. The current study tested the hypothesis that fetal genotype is an important determinant of ethanol teratogenesis by evaluating effects of ethanol exposure via embryo culture in three inbred strains of mice known to differ in the vulnerability of prenatal alcohol exposure *in vivo*.

Methods and results—Three strains of mice, C57BL/6N (B6), DBA/2 (D2), and 129S6/SvEvTac (129S6) were assessed in a whole embryo culture beginning on embryonic day 8.25 (E8.25), with or without alcohol administration at 88mM for 6 hours followed by 42 hrs culture in ethanol-free media. Contrasting strain differences in susceptibility were observed for the brain, the face, and other organ systems using the Maele-Fabry and Picard scoring system. The forebrain, midbrain, hindbrain, heart, optic vesicle, caudal neural tube, and hindlimbs of the B6 mice were severely delayed in growth, whereas compared to the respective controls, only the forebrain and optic vesicle were delayed in the D2 mice, and no effects were found in the 129S6 mice. A large number of cleaved(c)-caspase3 positive (+) cells were found in regions of the brain, optic vesicles, cranial nerve nuclei V, VII, VIII, and IX as well as the craniofacial primordial; only a few were found in corresponding regions of the B6 controls. In contrast, only a small number of c-caspase 3-im cells were found in either the alcohol-treated or the controls of the D2 embryos and in 129S6 embryos. The independent apoptotic markers TUNEL and Nile blue staining further confirmed the strain differences in apoptotic responses in both the neural tube and craniofacial primordia.

Conclusions—Under embryo culture conditions, in which alcohol exposure factors and fetal developmental staging were controlled, and maternal and intrauterine factors were eliminated, the degree of growth retardation and the extent and type of neurodevelopmental teratogenesis varied significantly across strains. Notably, the 129S6 strain was remarkably resistant to alcohol-induced

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growth deficits, confirming a previous *in vivo* study, and the D2 strain was also significantly less affected than the B6 strain. These findings demonstrate that fetal genotype is an important factor that can contribute to the variation in fetal alcohol spectrum disorder.

Keywords

fetal alcohol syndrome; embryo culture; mouse inbred strains; apoptosis

INTRODUCTION

The prevalence of fetal alcohol syndrome (FAS) (Ernhart et al., 1989, Jones and Smith, 1973, Stratton et al., 1996, Streissguth et al., 1991) is estimated to be about 1 per 1000 (Abel, 1995, Sampson et al., 1997, Stratton et al., 1996). Considering all children with alcohol-related neurodevelopmental disorder, regardless of whether they show the facial dysmorphology needed for the diagnosis of FAS, the prevalence of fetal alcohol spectrum disorder (FASD) may be as high as 1 per 100 (Sampson et al., 1997, Hoyme et al., 2005). Only about 5–10% of women who drink heavily during pregnancy give birth to children diagnosed with FAS (Abel, 1995; Stratton et al., 1996). Variability in functional deficits in cognitive, motor, social and emotional behavior are also evident across the spectrum of fetal alcohol disorders, with substantial individual differences in the severity of these effects (Jacobson et al., 1998, Streissguth et al., 1994, Streissguth et al., 1998). Why are some offspring of women who abuse alcohol during pregnancy severely affected, whereas others are not? To answer this question, it is important to understand the factors that contribute to the risk of prenatal alcohol-induced birth defects and neurodevelopmental disorder. Substantial evidence shows that one crucial factor is the variation in the alcohol exposure of the fetus resulting from differences in maternal drinking, i.e., quantity, frequency, pattern, timing, and duration of drinking during pregnancy (Abel and Hannigan, 1995, Coles, 1993, Jacobson et al., 1998, Maier and West, 2001). Another key factor is the developmental stage of the embryo or fetus at the time of alcohol exposure, which can determine the major outcomes of alcohol exposure, e.g., alcohol exposure during a relatively narrow temporal window on the seventh day of pregnancy in mice can induce craniofacial dysmorphology that models the craniofacial phenotype of FAS (Sulik et al., 1986).

In addition to variation in the dose, pattern, and timing of prenatal alcohol exposure, genetic differences have also been broadly implicated as important contributors to individual differences in FASD phenotypes (Riley and Lochry, 1982). However, the understanding of genetic contributions to the variation in alcohol-induced teratogenesis has been relatively limited to date. Genetic sources of variation in risk include effects related to differences in maternal genotype as well as differences in fetal genotype. Maternal factors, including maternal genetics, can influence the susceptibility to alcohol-induced fetal growth deficits and teratogenesis (Gilliam and Irtenkauf, 1990). Genetic differences in maternal alcohol metabolizing enzymes, e.g., alcohol dehydrogenase (ADH) isozymes, influence the risk for FAS (McCarver et al., 1997). Fetal genotype has also been implicated in the risk for FAS in a twin study (Streissguth and Dehaene, 1993), in that monozygotic twins were fully concordant for diagnosis (5 MZ twin pairs) whereas concordance for diagnosis of the dizygotic twins was 7/11. However, experimental analysis of genetic and environmental

factors is difficult in human studies because the alcohol exposure pattern cannot be controlled or manipulated experimentally, and actual alcohol exposure can only be estimated from drinking histories obtained in self-reported interviews.

Animal model studies, in which alcohol exposure can be manipulated and genetic and environmental factors can be controlled, have also implicated maternal and fetal genetic differences in susceptibility to adverse effects of prenatal alcohol exposure. For example, effects of offspring genotype have been implicated by evidence of differences in behavioral outcomes in selected lines of alcohol preferring (P) and non-preferring (NP) rats (Riley et al., 1993) and in high alcohol sensitive (HAS) and low alcohol sensitive (LAS) rats following early neonatal alcohol exposure directly to the pups (Thomas et al., 2000). Mouse models have been commonly used for studies of morphological damage induced by ethanol, particularly for teratogenic effects induced by high ethanol concentrations (Becker et al., 1996), and differences in effects among inbred strains of mice have been reported in various *in vivo* models. The C57BL/6 (B6) inbred strain consistently shows severe alcohol-related teratogenic effects, including fetal mortality, growth deficits, and skeletal, craniofacial, limb, kidney, ocular and CNS embryopathic malformations, when dams are given acute doses of 5–6 g/kg per day during the organogenesis period. Effects on the brain and behavior have also been reported in studies in which pregnant B6 dams consume alcohol in a liquid diet (Becker et al., 1996). In contrast, certain inbred strains, such as the DBA/2 (D2) strain, are significantly less susceptible (Boehm et al., 1997, Downing and Gilliam, 1999) or differentially respond to the teratogenic effects of ethanol in embryo culture models in which alcohol concentrations are controlled at equivalent levels between strains (Ogawa et al., 2005).

A recent study comparing the effects of alcohol (5.8 g/kg via maternal gavage on gestational day 9) in five different inbred strains of mice (Downing et al., 2009) reported that the 129S6/SvEvTac (129S6) strain was highly resistant to fetal weight deficits and malformations of the digit, kidney, brain ventricle, and vertebrae, effects that were significant in B6 mice. This study supports the contention that genetic differences among inbred strains critically determine the risk for teratogenesis, and comparisons between the pathogenesis mechanisms of susceptible strains (e.g., B6 mice) and resistant strains (e.g., 129S6 or D2 mice) can yield important insights into the genetic influences on prenatal alcohol-induced teratogenesis.

Whether the effects are mediated through maternal differences (including maternal genetics), through differences in prenatal (intrauterine) environmental factors, through fetal genetic differences, or combined effects from all three sources is a critical issue concerning the genetic contributions to the risk for alcohol-induced birth defects, even when comparing across different inbred strains. The present study tested the hypothesis that fetal genotype *per se* can contribute to the variability in alcohol-induced teratogenesis, by comparing the more vulnerable B6 inbred strain with the more resistant 129S6 and D2 inbred strains. We used an embryo culture model to assess the teratogenic effects of a 6 hour binge-like alcohol exposure initiated on embryonic (E) day 8.25 using culture methods that could closely match both the developmental staging and the alcohol exposure conditions for all embryos. Embryo culture eliminated the potential strain differences in the maternal factors and

intrauterine environment that are inherent with *in utero* exposure models and controlled the developmental staging of embryos that is difficult to achieve with *in utero* models even with carefully timed matings due to within- and between-litter variability in embryonic growth (Ogawa et al., 2005). The present study tested the prediction—derived from the findings of Downing et al. (2009)—that B6 embryos would show more severe alcohol-induced teratogenic outcomes than 129S6 or D2 embryos, both in terms of deficits in growth and morphology of the brain and somatic structures and in terms of the markers of alcohol-induced cell death (expression cleaved caspase-3; Nile blue staining; terminal deoxynucleotidyl transferase dUTP nick end labeling; (TUNEL) labeling of apoptotic cells).

MATERIALS AND METHODS

Whole Embryo Culture

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine (Indianapolis, IN) and are in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996).

The 12–14 week old C57BL/6N mice (average 20g) and DBA/2 mice (average 20g) were purchased from Harlan, Inc (Indianapolis, IN), and the 129S6/SvEvTac mice of similar age were purchased from Taconic Farm. Upon arrival, males were individually housed, while 5 females were housed together and were acclimated for at least one week prior to mating. Mice were maintained on a reverse 12:12 light: dark cycle (lights off at 0900), with ad libitum chow and water at 22°C room temperature, 30% humidity, in the Indiana University Laboratory Animal Research Center vivarium. Females were placed with one male for 2 hrs for mating, and vaginal plugs were checked after the mating period. When a plug was detected, it was designated as gestational day 0 (GD0) or embryonic day 0 (E0). On E8.2, animals were killed by CO_2 overdose and cervical dislocation. Uteruses were dissected out and removed. Decidual tissues and the Reichert membrane were removed carefully, leaving the visceral yolk sac and a small piece of the ectoplacental cone intact. To align the developmental stage, only embryos bearing 3–5 somites were used in the study.

Immediately after isolation from uteri, they were placed into a sterile container with 75% centrifuged heat inactivated rat serum (Harlan Sprague-Dawley, Inc., Indianapolis, IN) and 25% PB1 (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 8 mM Na2HPO4, 1.47 mM KH_2PO_4, 0.9 mM CaCl, 5.6 mM glucose and 0.33 mM sodium pyruvate; pH 7.4), with penicillin and streptomycin (20 units/ml and 20 microgram/ml, respectively; Sigma, St. Louis, MO). Containers were placed on a rotating culture system (B.T.C. Precision Incubator Unit; B.T.C. Engineering, Cambridge, England; 36 rpm) at 37°C and gassed with 5% O2, 5%CO2and 90% N2 for the first 24 hrs, and 20% O2, 5%CO2, and 75% N2 for the rest. After pre-culture for 1–2 hrs, all embryos of all strains were randomly assigned to either an alcohol treatment condition or a control (no-alcohol) condition. Embryos in the alcohol treatment condition were transferred into a medium containing 6 μl/ml of 95% ethanol and exposed for 6 hrs. We had previously determined that the media alcohol concentration was ~400mg/dL at the beginning of the treatment, and after 12 hrs (i.e., twice

longer in this study), it declined to ~300mg/dL, with no differences in the media alcohol concentration between B6 and D2 embryonic cultures (Ogawa et al., 2005). This target media alcohol concentration (peak ~400 mg/dL) is very similar to the blood alcohol concentration produced in dams following the intubation treatment (350–450 mg/dL) used by Downing and colleagues (Downing et al., 2009). The control group was cultured in the medium with no ethanol. The present study used a 6 hr alcohol exposure in culture, rather than the 44 hr exposure of the Ogawa et al. (2005) study, for two reasons. The 6 hr exposure allows a more precise analysis of the developmental time period in which various teratogenic effects can be induced, allowing a more precise analysis of temporal windows of vulnerability. In addition, the 6 hr exposure better matches a single episodic binge exposure in vivo that can facilitate comparisons to in vivo treatments at the same developmental period.

After the 6 hr alcohol treatment, culture media were disposed and changed back to alcohol-free culture media for both the control and treatment group. On day 2, embryos were transferred to fresh alcohol-free media 24 hrs after the initial culture. All the cultures were terminated 44 hrs after the initial culture (8.2+2). Yolk sacs were then opened, and embryos were fixed in 4% PFA at 4°C.

Morphological Scoring

A previously described morphological scoring system was used to quantitatively assess the morphological features of the mice embryos. The scoring system was adapted from Brown and Fabro (1981) and Van Maele-Fabry and Picard (1990) (Brown and Fabro, 1981, Van Maele-Fabry et al., 1992), and used in our previous report (Ogawa et al., 2005). The yolk-sac circulation, heart, caudal neural tube, forebrain, midbrain, hindbrain, otic system, optic system, branchial bars, forelimb, hindlimb and somites were individually scored, and a total score was given as a sum above the individual scores.

Non-parametric statistics were used for the morphological scoring (embryonic growth) analysis because the scores represented an ordinal classification scale. Kruskal-Wallis analysis, followed up by Dunn's *post hoc* tests, was used to test the strain differences in the total morphology score. Mann-Whitney U tests were used to test differences in each ROI between the control and alcohol-treated embryos of each strain. Group numbers were as follows: C57BL/6: Control n=12, Alcohol n=11; DBA/2: Control n=12, Alcohol n=9; 129S6/SvEvTac: Control n=7, Alcohol n=8. P-values of 0.05 and 0.01 (indicated by * and **, respectively) were used to indicate the degree of statistical significance. Statistics were performed using Prism Software Version 4 (GraphPad Software. Inc., San Diego, CA).

Immunocytochemistry

A monoclonal antibody against the specific form of intermediate apoptosis marker, cleaved-caspase-3 (c-caspase 3; activated form, cleaved adjacent to Asp175; Cell Signaling Technology, Danvas, MA, USA) was used which specifically recognizes the cleaved 18-kDa subunit of active caspase 3 and does not recognize the 32-kDa procaspase or other cleaved caspase3 (Olney, 2004). This antibody has been characterized by the preabsorption of antigenic peptide which completely blocked the ability of the antibody to recognize the

active c-caspase 3 in rat cerebellar cortices (Oomman et al., 2004). We have also performed a secondary antibody control in which the absence of the c-caspase 3 antibody resulted in negative staining. Further, the distribution of c-caspase 3-positive staining was found to be similar to that of apoptosis staining with Nile blue at the cranionucleus level (Dunty et al., 2002). In our immunocytochemical procedure, the alcohol-treated and the control embryos were embedded together in a single gelatin block and were carefully aligned by their level and orientation. Then, they were cut into 40μm sections using Leica VT 100S vibrating microtome and then processed free-floating in the same vial. They were treated equally in all aspects of the immunocytochemical processing from this point onwards. This practice avoids any bias through the staining procedure and is convenient for comparing levels of embryo sections side by side. Sections were incubated with 3% H_2O_2 in 0.1M phosphate buffered saline (PBS, PH7.4) for 10 min, washed in PBS, and then incubated in 1% Triton-X-100 in a phosphate buffer overnight. Sections were preincubated in PBS containing 0.1%Triton-X, 1.5% normal goat serum for 90 min before incubation with anti-c-caspase-3 antibody (Rabbit polyclonal, 1:150, Cell signaling, Beverly, MA) overnight. The next day, sections were washed three times in PBS and then incubated with a biotin-conjugated Goat-anti-Rabbit secondary antibody (1:500, Jackson ImmunoResearch Lab, West Grove, PA) for 90 min. Sections were rinsed in PBS and then incubated in peroxidase-conjugated streptavidin (1:500, Jackson ImmunoReseach Lab, West Grove, PA) for 60 min. The PAP reaction was performed with 0.003% H_20_2 and 0.05% 3′ 3-diaminobenzidine (Sigma, St. Louis, MO) for 10 min. The primary, secondary, and third antibodies were diluted in PBS containing 0.1% Triton X-100 and 1.5% normal goat serum. All procedures were done at room temperature. All sections were Nissl-counterstained with methyl green to reveal background cells to identify embryo structures.

Nile Blue Vital Staining

Nile blue (NB) staining was performed to stain apoptotic nuclei, which are seen in cells that died by apoptosis. B6 and 129S6 embryos (n=4–5 pairs of control and alcohol-treated) were examined at around E10.2. Nile blue (1:10,000 in PBS, Sigma, St. Louis, MO) was placed into culture media 2 hrs before the culture ended (Kotch and Sulik, 1992). Embryos free of a yolk sac were stained for 2 hrs at 37°C, followed by a washing in PBS for 15 min, and then fixed in 4% PFA. Both alcohol-treated and control embryos were processed simultaneously, allowing for an accurate comparison of patterns of cell death. Photographs were taken immediately after fixation under stereomicroscope (Leica MZFLIII). NB staining distributions were comparable to previous NB staining in embryos by Dunty et. al. (Dunty et al., 2002).

TUNEL Staining

TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining labeling DNA strand breaks was performed to detect apoptotic cells. TACS™ 2TdT-DAB in situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) was used. Gelatin- embedded embryo sections were prepared as above. Staining was performed following the kit's instructions. In brief, tissue sections were permeablized with proteinase K (1:200) for 30 min at room temperature, followed by incubation in 3% hydrogen peroxide in PBS for 5 min to quench endogenous peroxidase. Then, a labeling reaction mix (containing dNTP mix

1:50, cation buffer, TdT enzyme 1:50 in labeling buffer) was applied at 37°C for 1 hr, followed by washing with a stop buffer and transferring to strep-HRP solution for 10 min at 37°C. Sections were then washed in PBS, developed for coloration in peroxidase substrate with 0.05% DAB and 0.03% H_20_2, and counterstained with methyl green. A positive control treated with DNase (TACS-Nuclease, 1:50, Trevigen, Gaithersburg, MD) was included in each experiment, which also indicates the specificity and sensitivity of TUNEL labeling.

Quantification of c-caspase 3+ and TUNEL+ Cells

The assessment for c-caspase 3 + cells and TUNEL+ cells were measured by staining density using NIH imaging (Bethesda, MD). Images were taken using a Leitz Orthoplan II microscope and a high-resolution SPOT2 camera (under 6.3X or 10X, depending on the size of structures measured, in a bright field). All the images were taken under the same setup with the same exposure time. The density of immuno-labeled or TUNEL-labeled cells in a specific tissue area was compared between the alcohol treated group and the control group in three different mice strains. Images were converted into 16-bit color in ImageJ. A threshold value was set up to eliminate noise from the signal and to subtract the background. The same value was applied consistently to all the images. Signals above the threshold in ROI (region of interested) were measured by integrated density using the ImageJ analysis tool. The density value of consecutive sections was added up and constituted the region of interest. The total 14 regions of embryos between the alcohol and control groups were measured according to *The Atlas of Mouse Development* (M.H. Kaufman, *1992*), including the central nerves system, peripheral nerves system, cardiac system, limbs and somites. For c-caspase 3 staining analysis, data were analyzed using two-way ANOVA with the treatment group and strain as between-subjects factors. C57BL/6: n=5; DBA/2: n=5; 129S6/SvEvTac: n=5. The significance of the treatment effects were followed up with a Bonferroni *post hoc* test. In addition, using Tukey's *post hoc* test, one-way ANOVAs were used as a follow-up comparison among controls of three strains of embryos. An alpha level of 0.05 was used to assess significant results. For TUNEL staining analysis, a t-test was used for a comparison between alcohol treated and control embryos. C57BL/6: n=4; 129S6/SvEvTac: n=3. Data are presented as Mean ± standard error means (SEM). All statistics were performed using Prism Software Version 4 (GraphPad Software. Inc., San Diego, CA).

RESULTS

Growth Delay Analysis

The basal development of the three strain embryos was similar as indicated by the Brown and Fabro (1981) and Van Maele-Fabry and Picard (1990) growth scoring system; there was no difference on the total score of the Control embryos among the three strains ($P>0.05$, Kruskal-Wallis test). In contrast, the total scores of the Alcohol-treated embryos were significantly different among strains (Kruskal-Wallis value =7.803, $p<0.05$). The overall score indicated that there was a significant alcohol-induced delay of B6 and D2 embryos relative to their controls, with greater delay in B6 mice; the 129S6 mice showed no evidence of alcohol-induced growth delay (Table 1, Mann-Whitney U tests). For B6 mice, the growth rate was significantly retarded in the Alcohol-treated group as compared to the Controls, and significant reductions were seen in the heart (Table 1), fore-, mid-, and hind-brain, caudal

neural tube, optic vesicle, and hindlimb (Figure 1A, B). For the D2 mice, alcohol-induced growth delay was seen only in the forebrain and optic vesicle (Figure 1C). In contrast, the 129S6 mice demonstrated remarkable resistance to alcohol exposure; no significant growth delay was observed (Figure 1D).

Apoptosis Analysis

Assessing from different levels, the three apoptosis analyses, the cleaved caspase 3, TUNEL, and Nile blue agreed on differential apoptotic vulnerability across the strains:

a. Cleaved Caspase 3—In B6 Control, the c-caspase 3 positive ($^+$) cells appeared sporadically in the fore-, mid-, and hindbrain, clustered in low density in the trigeminal (V) ganglia, facial (VII) and acoustic (VIII) ganglion complex, glossopharyngeal (IX) and vagus (X) nerve ganglion, and dorsal root ganglia between somite 5 and 28. In the Alcohol-exposed B6 group, a vast increase of c-caspase 3^+ cells were seen throughout the fore-, mid-, and hindbrain, except for the frontal pole of the forebrain (Figure 2A, B). Thus, the increase of c-caspase 3^+ cells was not only in the area of natural apoptosis [e.g. cranial nucleus V, VII–VIII, & IX–X, and DRG and associated somites (Figure 2C, D)], but also in new brain regions that do not normally show apoptosis [e.g., a distinct group of c-caspase 3^+ cells was prominent along the midline (Figure 2B)]. Noticeable increases of c-caspase 3^+ cells were also seen at rhombomere (r) 1 and r2 that appear in the rostral and ventral subventricular zone of the fourth ventricle. Other than brain regions, the c-caspase 3^+ cells also increased in the heart. Importantly, the craniofacial regions are keys to distinguishing the strain difference in differential apoptosis. In the B6 mice, alcohol increased c-caspase 3^+ cells in the forehead region and branchial arches I and II of the Alcohol-treated groups as compared with Controls (Figure 3).

In 129S6 Control, fewer c-caspase 3^+ cells were found as compared with those of B6. Alcohol did not significantly increase c-caspase 3^+ cells in most of the brain or craniofacial areas, except for the three cranial nuclei V, VII and VIII, where a modest increase was seen and measured (Figure 2I–L).

Similarly, in D2 Controls significantly fewer c-caspase 3^+ cells were seen as compared with those of B6 Controls. Alcohol did not induce a detectable increase in c-caspase 3^+ cells in the D2 embryos, except in the heart (Figure2E–H). Craniofacial c-caspase 3^+ cells were not significantly increased by alcohol treatment either in D2 or in S129S mice.

A quantification of c-caspase 3 staining optic density in the above regions is shown in Figure 4. Two-way ANOVA showed a significant interaction among the three strains and treatment in cranial nuclei V [$F(2, 23)=3.973$, $P<0.05$], VII & VIII [$F(2, 17)=5.533$, $P<0.05$)], IX & X [$F(2, 23)=8.715$, $P<0.01$)], heart [$F(2, 23)=4.45$, $P<0.05$], rhombomere (r1–r2) [$F(2, 23)=3.654$, $P<0.05$)], 1st branchial arch [$F(2, 17)=4.838$, $P<0.05$], and optic vesicle [$F(2, 23)=5.032$, $P<0.05$)], indicating significant differential effects of alcohol treatment on these three strains. Strain comparisons indicated that in B6 embryos, significant differences between the alcohol and control groups were seen in the heart, forebrain, and caudal neural tube, as well as the craniolfacial regions, including trigeminal (V) nucleus, VII & VIII, and IX & X nuclei, and otic vesicle (Figure 4). In D2 embryos, only the heart

(P<0.02) showed a significant difference between the alcohols and controls. In 129S6 embryos, cranial nerve nuclei V (P<0.05) and VII–VIII (P<0.05) showed significant differences. When comparing B6 with 129S6 using a two-way ANOVA, significant alcohol treatment x strain interactions were shown in nucleus VII–VIII [F(1,11)=11.16, P<0.01], cranial nucleus IX–X [F(1,11)=20.33, P<0.005], heart [F(2,15)=5.51, P<0.05], rhombomere (r1–r2) [F(2, 15)=5.680, P<0.05], optic vesicle [F(2,15)=19.22, P<0.005], and 1st branchial arch [F(1,11)=5.821, P<0.05]. Likewise, when comparing B6 with D2, VII–VII [F(1,11)=6.266, P<0.05], IX& X [F(1,11)=7.561, P<0.05], and heart [F(1,11)=5.311, P<0.05] showed significant alcohol treatment x strain interactions, indicating the effects of alcohol identified in these regions depended on the two fetal genotypes. However, there was no significant interaction of alcohol treatment x strain when comparing D2 and 129S6 strains in any of the above regions except for the caudal neural tube. Furthermore, one-way ANOVA for Controls of all the three strains showed only one region with a significant difference, the midbrain [F(2, 10)=5.97, P<0.05]. This suggests there were few baseline differences in c-caspase 3 expression across D2 and 129S6 strains. In contrast, one-way ANOVAs for the alcohol groups of the three strains showed significant strain differences in most of the regions.

b. Nile Blue—Nile blue vital staining was performed in the two contrast strains, B6 and 129S6.

In B6 mice, at about E10.25, the telencephalic vesicles and fourth ventricle were well differentiated; a scattered pattern of moderately stained Nile blue positive (NB+) cells was observed at this stage in the forebrain and midbrain in the control embryo. Ethanol treatment resulted in a significant increase in NB staining with more NB+ cells evident in the regions identified above (Figure 5). Specifically, a dramatic increase in the dorsal midline of the forebrain was consistently seen (Figure 5D).

Cranifacial features including distinct first and second branchial arches, migrating neural crest cells, and cranial nerve ganglia are visible through NB staining. More NB+ staining was present along the proximal rim of the mandibular prominence and second branchial arch, and at the junction between maxillary and mandibular prominence. This is consistent with a previous report (Dunty et al., 2002). In the cranial nuclei, increased Nile blue uptake was observed in the cranial nuclei V, VII & VIII, and IX, and neural crest cells migrating to the third branchial arch. Furthermore, an increase of NB uptake from moderate or no staining to heavy staining were found in the olfactory placode, peripheral boundaries of the olfactory pit, optic vesicles, and Rathke’s pouch of alcohol exposed embryos as compared to their counterparts in the controls. Particularly, an excessive increase of NB uptake was seen in the optic stalk of the alcohol group. Also at this stage, NB staining was seen mostly in somite regions rostral as well as the caudal to forelimb, i.e. somites 5–7 and 9–13. The forelimb region always takes up dye in both the control and ethanol-treated embryos, though the ethanol-treated embryo showed more uptake in the distal end of the forelimb. Also, where severe malformation was found in the caudal neural tube, Nile blue was also intensified at the region including lower limbs.

In 129S6 mice, overall NB uptake was less than that in B6 mice in both the control and ethanol-treated embryos. No increase of staining or number of NB+ staining in the fore- mid- or hindbrain was seen in the alcohol-treated group as compared to the 129S6 controls. Maximal Nile blue uptake was observed in the forebrain midline, olfactory pit, mandibular prominence of the first branchial arch, forelimb, and rostral somites. Mild increase of NB staining could be seen in cranial nuclei V, VII & VIII., olfactory placode surrounding the pit, maxillary and mandibular prominence after alcohol treatment (Figure 5G–M).

c. TUNEL staining—Confirmation of the caspase 3 for apoptosis with TUNEL staining was demonstrated in B6 and 129 embryos (Figure 6 and Table 2) as compared with caspase3 (Figures 2 and 3). In B6 embryos, an overall significant increase of TUNEL positive cell was seen in the forebrain ($P<0.001$, t-test, $N=4$) and midbrain ($P<0.05$), as well as cranialfacial features, including V ($P< 0.01$), VII–VIII cranial nuclei ($P<0.005$), 1st branchial arch ($P<0.01$). The somites and dorsal root ganglia at the level of forelimb ($P<0.05$) also showed more TUNEL positive cells in alcohol-treated embryos as compared to those of Controls (Figure 6C, D). In 129S6 embryos, most of the above regions do not show significant increase of TUNEL+ cells, except VII–VIII cranial nuclei ($P<0.05$) (Table 2).

DISCUSSION

The major goal of the current study was to determine whether fetal genotype is an important determinant of susceptibility to prenatal alcohol-induced birth defects. We tested the hypothesis that previously identified strain differences in teratogenesis (Downing et al., 2009), resulting from heavy *in utero* alcohol exposure, would be replicated by binge-like alcohol exposure directly to the embryos via embryonic culture. As predicted from the *in utero* models showing significant and severe alcohol-induced growth deficits and structural malformations (fetal weight deficits and kidney, brain ventricle, and vertebral malformations) (Downing et al., 2009), the present study confirmed major alcohol-induced teratogenesis in the B6 mice and further demonstrated that apoptotic cell death was evident, together with significant growth retardation, in many brain regions, craniofacial structures, the heart, and the neural tube. The present study also confirmed that the 129S6 mice were remarkably resistant to alcohol-related growth deficits, and the D2 strain showed alcohol-induced growth deficits only in the forebrain and optic regions. This study provides new evidence that alcohol-induced apoptotic cell death was also greatest in the B6 strain, whereas the 129S6 strain showed significant increases in c-caspase 3+ cells only in cranial nerve nuclei V, VII and VIII, and the D2 strain showed no evidence of alcohol-induced increase in c-caspase 3 expression.

These differences in the extent of alcohol-induced teratogenesis in embryo culture provide conclusive evidence that fetal genotype is an important contributor to susceptibility to alcohol-related teratogenesis. Embryo culture allows control over major non-genetic factors including alcohol exposure variables and alignment of the developmental stage at the time of exposure, and it eliminates maternal physiology, metabolism, and genetics, along with other sources of non-genetic variation (e.g., intrauterine environment). In this model system, significant strain differences in outcomes can be directly attributed to genetic differences

between the strains. Moreover, the alcohol exposure was clinically relevant in that it models a single heavy binge exposure at the time of neurulation that was limited to six hours and was comparable across the three inbred strains. These differences in embryonic effects in a pharmacologically relevant exposure provide a useful model to pursue a systematic search for genes (or alcohol-regulated gene expression) in the embryos that impart relatively susceptibility or resistance to the teratogenic effects of alcohol. Although the effects reported here reflect effects of a single exposure at a relatively high concentration in an embryo culture model that lacks potentially important maternal and intrauterine effects that may be critical for effects on alcohol effects *in vivo*, these findings do predict that strain differences in the dose-response of growth deficits and of adverse outcomes on brain and behavioral deficits would be evident between the B6 and 129S6 strains.

Differential Dysmorphology

The current study confirmed that the embryonic growth deficits induced by the 6 hr alcohol treatment differed significantly among the inbred strains, with more retardation in the mid- and hindbrain, caudal neural tube, heart, and optic vesicle in B6 than D2 or 129S6 mice. The differences between B6 and D2 mice are consistent with our previous study that used a 44 hr alcohol treatment (Ogawa et al., 2005), though the 44 hr alcohol treatment of that study did produce a delay in heart formation in D2 mice—an effect not observed with the 6 hr exposure of the current study. The current results indicate that the 6 hr binge-like alcohol exposure at the beginning of neurulation is critical in causing delays in growth of many of the above organ structures. Furthermore, 6 hrs of alcohol exposure at this early embryonic stage was sufficient to reveal the differential effects on growth among the three strains. In particular, the 129S6 was resistant to the 6 hr alcohol exposure of the current high dose of alcohol; since that strain was not included in the previous study, we do not have data from a more prolonged alcohol exposure (44 hrs) to assess the impact of a longer duration of exposure. Our current findings showing major teratogenesis in the B6 mice and relatively limited effects in the 126S6 mice are also in agreement with the findings of Downing and colleagues (Downing et al., 2009), who reported that administration of 5.8 g/kg ethanol via gavage to pregnant dams on E9 caused extensive teratogenesis, including fetal weight deficits and kidney, brain ventricle, and vertebral malformations in B6, but not in 129S6.

Though the presence of c-caspase 3, an active form of caspase 3, does not necessarily indicate final cellular commitment to apoptosis [e.g., antibody may detect caspase 9-like component (Fan and Bergmann, 2010)], it has been compared with TUNEL with good fidelity in detecting programmed cell death (Duan et al., 2003). Our study indicated that the c-caspase 3 has good correlation with Nile blue (Figure 2, 3, and 5) and TUNEL staining (Figure 2, 3, and 6) in early development. The severe alcohol-induced apoptosis found in the brain regions, heart, limb, somite, and dorsal root ganglia in B6 embryos, compared to the much lesser degree in 129S6 or D2 embryos, demonstrated differential vulnerability of the three strains to alcohol exposure. Besides the above regions where growth delay occurred (e.g. forebrain, midbrain, heart), additional evidence of extensive apoptosis in B6 but not in D2 strains was present in the brain midline, craniofacial region (including forehead, and first, second, and third branchial arches), truncal dorsal root ganglia, and in major cranial nuclei (V, VII, and VIII). The 129S6 strain showed an increase of apoptosis in V, VII and

VIII cranial nuclei but not in other regions. To the extent this apoptosis reflects or predicts enduring reductions of the cells within an organ, it supports the findings of growth delays in the corresponding regions, e.g. fore-, mid-, and hind-brain, and would also contribute to the facial dysmorphology and neurodevelopmental deficits that are diagnostic for FAS.

Strain Considerations

Although the genetic differences among the inbred strains that underlie the differential vulnerability are yet to be investigated, given the striking contrast in susceptibility to alcohol-induced teratogenesis, it is interesting to note the three stains are derived from diverse backgrounds. The B6 and D2 strains are well known for their extensive genetic and phenotypic differences. B6 mice are generally referred to as alcohol-preferring and D2 as alcohol-avoiding (Le et al., 1994). They differ not only in their alcohol drinking propensity but also in response to alcohol and cocaine stimulation (Fish et al., 2010, De Waele et al., 1992) and their insulin secretion in response to a high fat diet (Andrikopoulos et al., 2005). The current study adds the embryonic vulnerability to alcohol to the catalogue of differences between B6 and D2 mice.

The B6 and D2 strains are widely used and the 129S6 strain to lesser degree in alcohol-related studies or in other research studies, yet generalization of effects, including teratogenic effects, from studies of a few inbred strains can only be made with caution. For example, teratogenic effects observed with inbred strains are often either limited when assessed in genetically heterogenous stocks or show dominance toward resistance to teratogenic effects in outbred crosses (Gilliam et al., 1988, Gilliam and Irtenkauf, 1990). In addition, comparison of differences involving only a few inbred strains are inadequate to assess genetic correlations of various phenotypes; for example, the B6 and D2 strains not only differ in alcohol preference and resistance to alcohol's teratogenic effects but also in the rate of progression through development staging. Inferences concerning genetically correlated phenotypes cannot be made from comparisons of only a few inbred strains, but the clear demonstration of strain differences in embryos of these three strains does provide a starting point for more mechanistic studies of genetically-based susceptibility and resistance.

The 129S6 (129S6/SvEvTac) strain, which was recently characterized with respect to fetal alcohol vulnerability (Downing et al., 2009), is derived from a congenic strain made by out crossing to introduce the *Steel* mutation. The expression of the *Steel* locus is essential for normal development of three populations of stem cells: the neural crest-derived melanoblasts, germ cells, and blood cell precursors (Keller et al., 1990). All existing 129 mouse inbred substrains have a 25bp deletion in exon 6 of Disc1 (disrupted in schizophrenia 1) gene (Clapcote and Roder, 2006) which induces a frame shift in the reading frame of *Disc1*, resulting in 13 novel amino acids, followed by a premature stop codon. It is unclear if such deletion affects neuronal characteristics. There are currently more than 15 substrains of the 129 lines, and a large degree of genetic diversity has been identified among the 129 substrains (Simpson et al., 1997). The apoptotic resistance and invulnerability of 129S6/SvEvTac to alcohol exposure should not be generalized with the other 129 substrains yet.

Another point of interest in light of current finding is that virtually all embryonic stem (ES) cell lines for gene targeting are derived from substrains of the 129S6 mouse. It is highly

likely these stem cells would be subject to similar resistance. Researchers using these three strains of mice for studies of plasticity and developmental studies must be cautious in keeping the current finding in mind.

Mechanistic Consideration

The mechanisms behind the differential embryonic vulnerability are unknown. There are no significant differences in gene expression of alcohol dehydrogenases (ADH), catalase, and a number of the cytochrome P450 enzyme family between B6 and D2 mice; aldehyde dehydrogenase 2 (ALDH2) and aldehyde dehydrogenase 1A1 (ALDH1A1) are higher in D2, whereas aldehyde dehydrogenase 7A1 (ALDH7A1) is higher in B6 (Bhave et al., 2006). One report indicated that hepatic and brain catalase activities were two- to three-fold higher in adult D2 than in adult B6 mice (He et al., 1997). Alcohol dehydrogenase 3 (ADH3) is a major form of alcohol catabolic enzyme in embryonic alcohol metabolism (Duester, 1994). Information on the expression of ADH3 in the embryonic B6, D2, and S129S is not yet available. If the observed strain differences are related to tissue-specific differences in expression of alcohol-metabolizing genes, then filling the gap of knowledge will be an important step toward understanding fetal genotype's risk for FASD.

The current study is the first demonstration that the alcohol-induced differential vulnerability of embryos from three different inbred strains of mice could be distinguished at the cellular level with differential apoptosis. This is an important step toward a better understanding of the cellular mechanisms for differential vulnerability at the genetic level. We demonstrated that caspase 3 is not as extensively induced by alcohol in 129S6 and D2 embryos as compared to B6 embryos. It is possible that 129S6, for example, may not use the caspase-dependent pathway for programmed cell death (Momoi et al., 2003), or its caspase-dependent pathway was less sensitive to alcohol.

Significance and Application

In summary, we found that the binge alcohol exposure at early neurulation resulted in strikingly different effects in growth retardation and apoptosis among the B6, D2, and 129S6 inbred strains. The differential vulnerability was not only evident in the brain and heart, but also in the embryonic facial structures, including the maxillary, mandibular arch, olfactory placode, and optic vesicle. The varying degrees of vulnerability, ranging from severe to partial to none in growth retardation and apoptosis are attributable to genetic differences between the strains rather than to the variability in alcohol exposure (amount, concentration, pattern, timing, or duration), in the embryonic stage of development or in maternal factors (metabolism or intrauterine physiology). This study definitively demonstrates that the fetal genotype is an important contributing factor to the risk for birth defects induced by binge-like alcohol exposure during early neurulation. The variable spectrum of prenatal alcohol-induced brain abnormalities and facial dysmorphology has been observed in FASD populations of different ethnic groups, e.g. Italy, South Africa, and Finland (Ceccanti et al., 2007). The current observation provides biological evidence and support that the diverse response to binge-like prenatal alcohol exposure in altering development of the brain and face can depend, in part, on the genotype of the offspring. It is important to identify the specific genes that impart susceptibility or resistance to alcohol exposure. Identifying those

genes would provide insight into a better understanding of the mechanisms by which alcohol causes growth retardation and apoptosis, and it could potentially provide biomarkers for individual susceptibility to or resistant against the alcohol insult.

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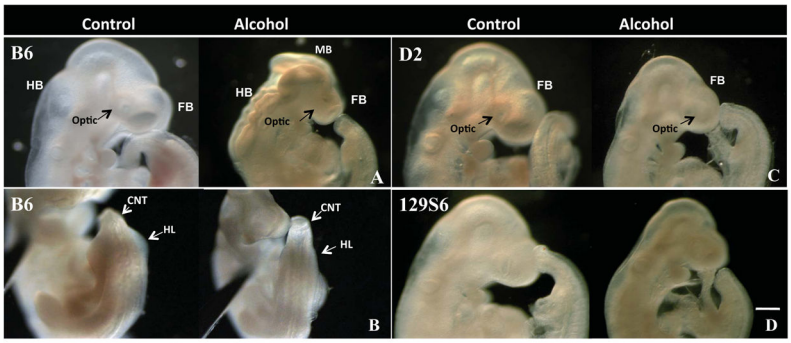

Figure 1.

Morphology of control and alcohol-treated embryos (E8.2+2) in three strains: B6, D2 and 129S6. Neural tube-opening was seen in forebrain, midbrain, hindbrain (A right panel) and caudal neural tube of B6 embryo (B, right panel). In contrast, the growth retardation was not apparent in D2 (C) or in 129S6 (D) as compared between their Control and alcohol-treated embryos respectively. FB: forebrain; MB: midbrain; HB: hindbrain; CNT: caudal neural tube; HL: hindlimb. Scale bar: A–C=300μm.

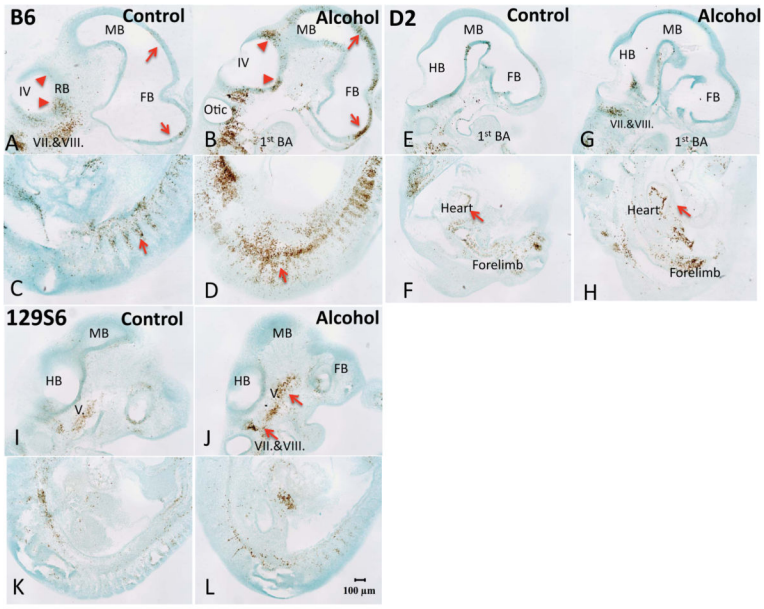

Figure 2.

The Contrast expression of c-caspase 3 + cells upon alcohol treatment in B6 (A–D), versus D2 (E–H) and 129S6 (I–L) E8.2+2 embryos. Midsagittal sections (A, B) showed a vast increase of c-caspase 3 + cells in alcohol-treated embryos throughout fore-, mid-, and hind-brain, cranial nucleus V, VII–VIII, X, and dorsal root ganglia cells along with somites (C, D). An increase of c-caspase 3^+ cells was seen prevalent at the midline along forebrain (A, B, arrow). A noticeable increase of c-caspase 3^+ cells was seen at the rostral and ventral sub-ventricular zone of the 4th ventricle (A, B, arrowheads). A similar distribution of c-caspase 3^+ cells in control B6 was seen in the D2 and 129S6 control. No noticeable increase of c-caspase 3^+ cells was seen in most parts of the brain in alcohol-treated D2 and 129S6 as compared to their respective control groups, except for a minor increase in cranial nuclei V and VII& VIII cranial nerve cells of alcohol-treated 129S6 (J, arrow). FB: Forebrain; MB, midbrain; HB: hindbrain; NT: neural tube. Scale bar: A–L=100µm.

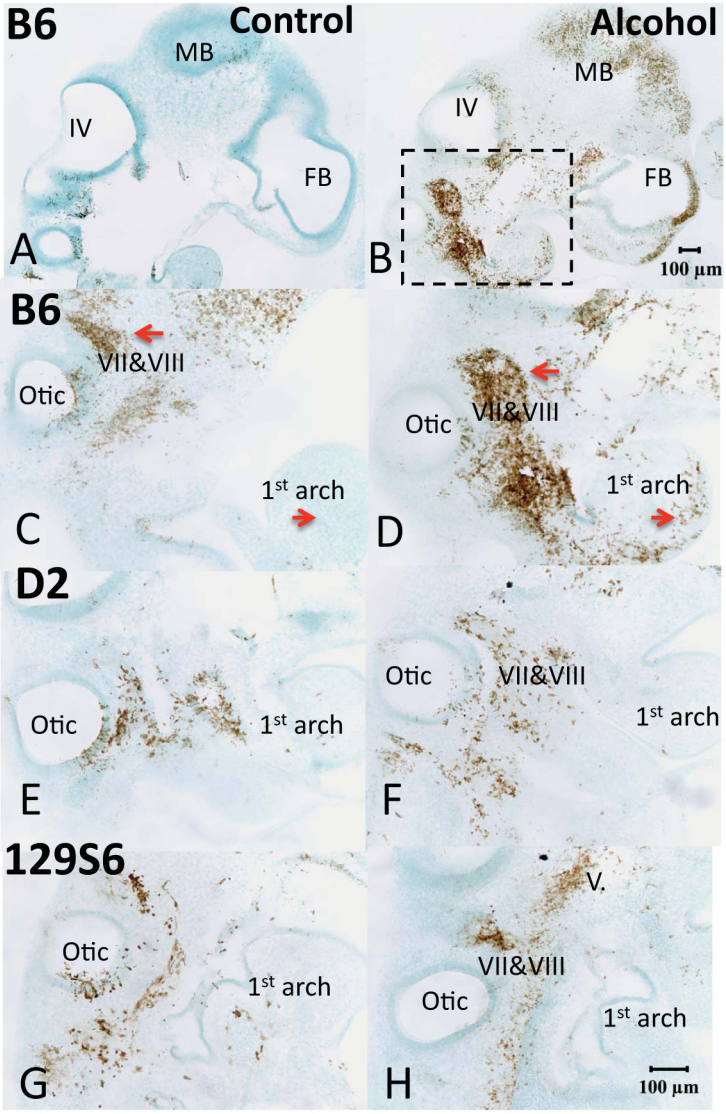

Figure 3.

Distribution of c-caspase 3+ cells in craniofacial and metencephalic regions of B6, D2 and 129S6 embryos in para-sagittal section. Alcohol-treatment increased extensively the caspase level and number of c-caspase 3+ cells in the local structure of these regions in B6 (A, B). Higher magnification of boxed region in B showing significant increase of c-caspase 3+ cells in cranial nuclei VII& VIII cranial nerve cells in alcohol-treated embryo was shown in D. Scale Bar: A, B=100μm, C–H=100μm.

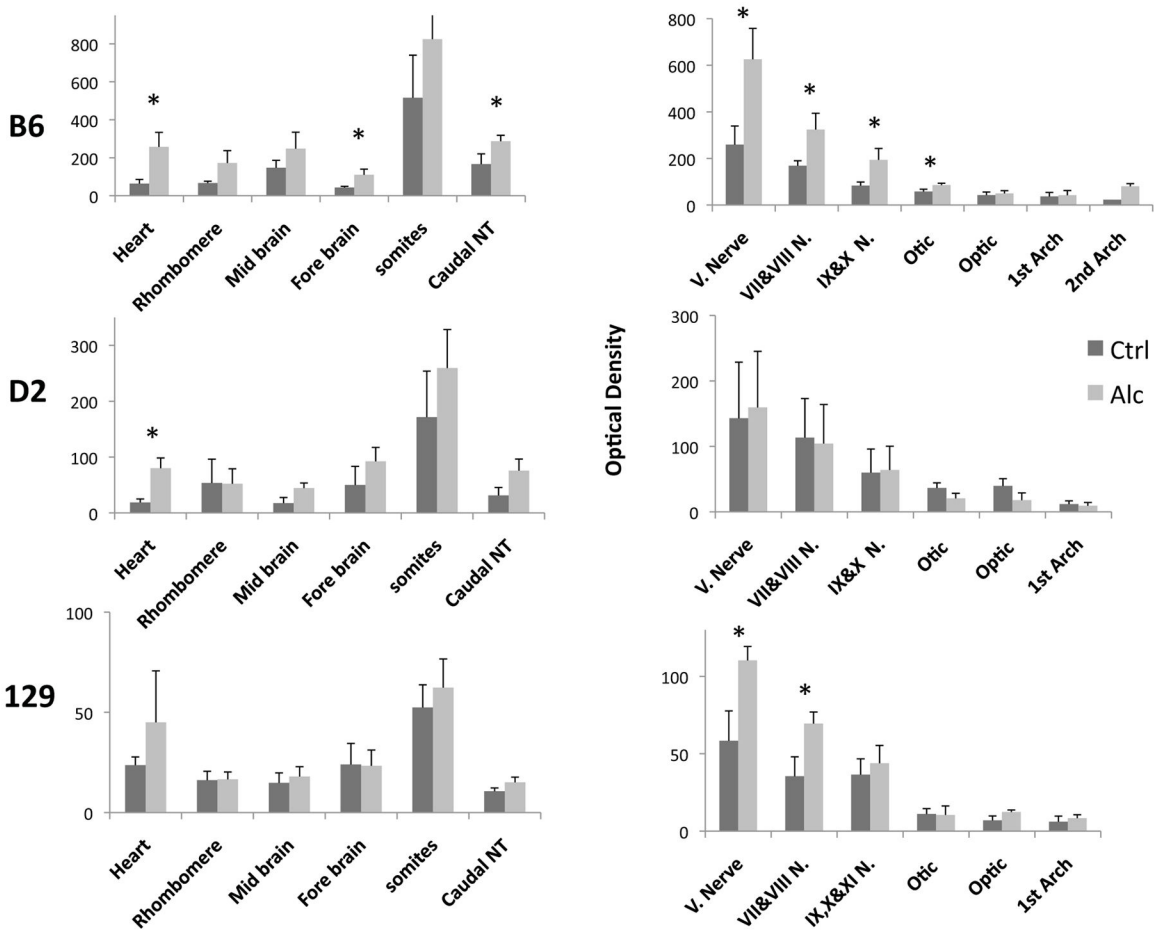

Figure 4.

Semi-quantification of c-caspase 3+ cells in B6, D2 and 129S6 embryos. 12 ROIs were included: left panels showing brain, heart, caudal neural tube and somites; right panels showing craniofacial features of each strain. N=5, *P <0.05, data presented as Mean ± SEM. Major increase of caspase distribution density was seen in B6. Significant alcohol effects were present only in the heart of D2, and cranial nuclei of S129S6.

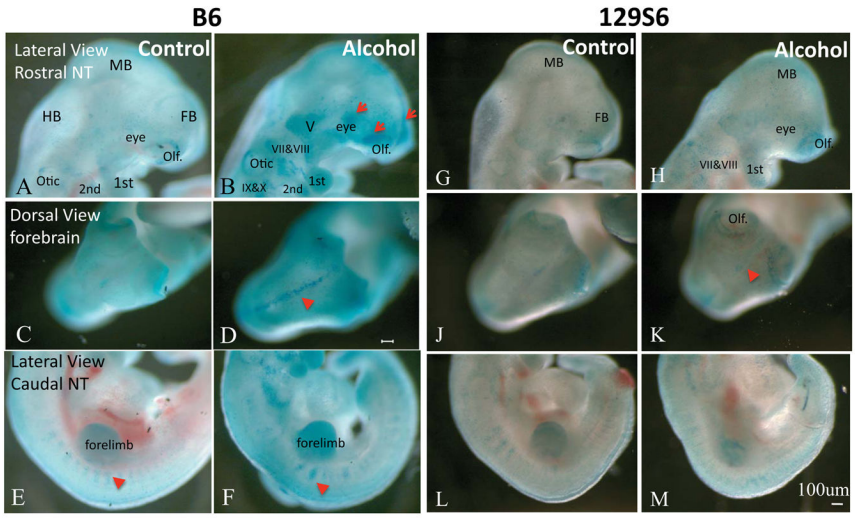

Figure 5.

Nile Blue (NB) staining was increased in eye, olfactory pit, midbrain, otic stalk, 1st and 2nd branchial arch, VII&VIII nerve- and IX, X cranial nerve nuclei of alcohol treated B6 as compared to its control (A, B). A conspicuous increase of NB staining was shown in midline of forebrain of alcohol treated B6 embryo (D, arrowhead), similar to that of c-caspase 3 (Figure 2); an increase in NB was also seen in somites at level of forelimb (E, F). No apparent difference of NB staining was seen in the 129S6 control and alcohol embryo (G–M). Arrowhead: forebrain midline (D, K) and dorsal root ganglia in somites (E, F). Olf.: olfactory pit. Scale bar: A–M=100μm.

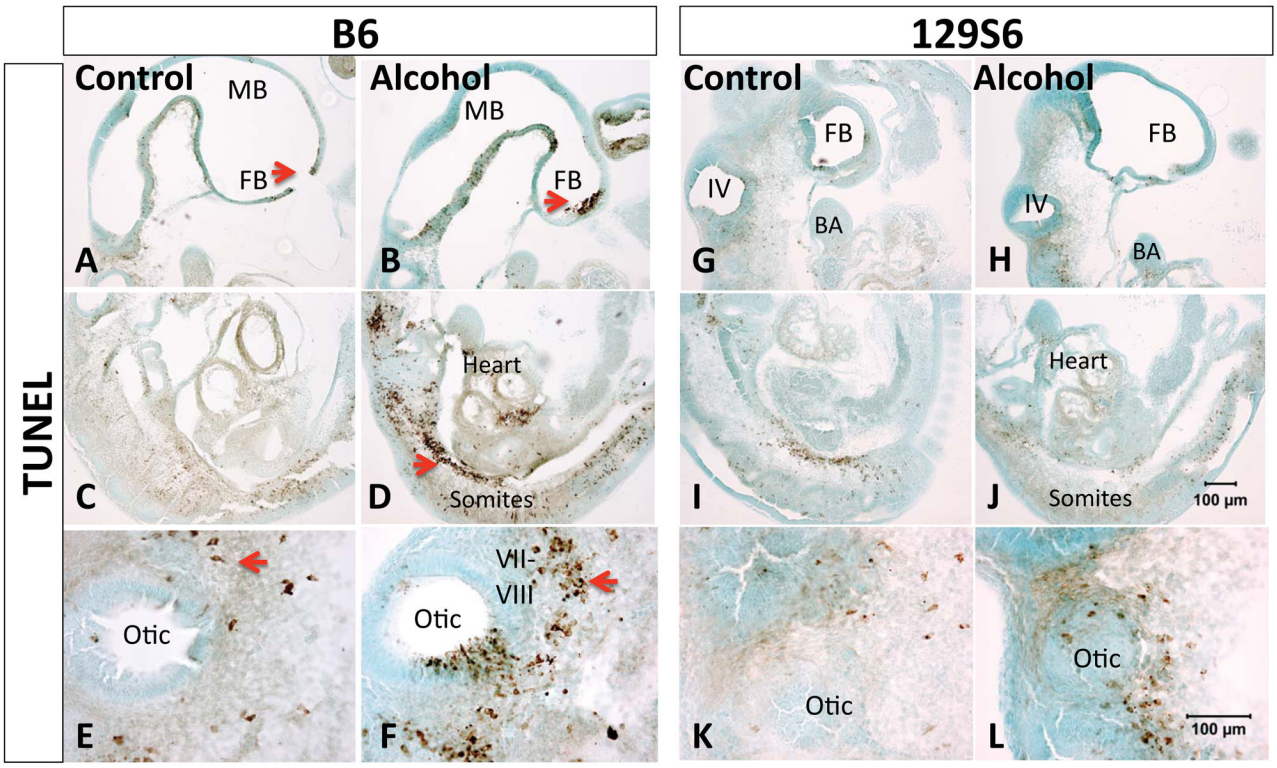

Figure 6.

The TUNEL staining is shown here with similar distribution to that of c-caspase 3 (Figure 2 and 3) in the B6 and 129S6 embryos. More TUNEL+ cells were found in alcohol-treated (B, D) than those in Control embryo (A, C). Higher magnification of boxed region in A and B was shown in E and F respectively, indicating more TUNEL+ cells in cranial nuclei V, VII, & VIII, and in first and second branchial arches after alcohol treatment. Scale bars: A, B, C, D= 100µm; E, F=100µm.

Table 1

Morphological scoring of growth rate of B6, D2 and 129S6 embryos after 6hr 88mM alcohol treatment.

	C57/BL6		DBA/2		129S6/SvEvTac	
Region	**Control**	**Alcohol**	**Control**	**Alcohol**	**Control**	**Alcohol**
Yolk sac circulatory system	4.00±0.00	3.64±0.15	4.00±0.00	3.78±0.15	4.00±0.00	4.00±0.00
Allantois	3.00±0.00	2.91±0.09	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
Flexion	5.00±0.00	4.27±0.03	4.75±0.13	4.67±0.17	4.71±0.18	4.75±0.16
Heart	4.83±0.11	4.00±0.27*	4.75±0.13	4.33±0.29	4.57±0.20	4.63±0.26
Caudal neural tube	4.92±0.18	4.18±0.23*	4.92±0.08	4.89±0.11	5.00±0.00	5.00±0.00
Hind brain	4.75±0.13	4.09±0.21*	4.92±0.08	4.56±0.18	5.00±0.00	4.88±0.13
Midbrain	4.92±0.08	4.09±0.25*	4.92±0.08	4.44±0.24	5.00±0.00	5.00±0.00
Forebrain	5.17±0.17	4.00±0.23**	5.08±0.15	4.44±0.18*	5.00±0.00	5.00±0.00
Otic vesicle	4.17±0.11	3.91±0.31	4.33±0.14	4.11±0.11	4.43±0.20	4.38±0.18
Optic vesicle	3.75±0.13	3.09±0.21*	3.75±0.13	3.11±0.20*	4.43±0.30	4.88±0.13
Olfactory system	0.58±0.15	0.55±0.16	0.50±0.15	0.44±0.18	0.00±0.00	0.00±0.00
Branchial bars	2.83±0.11	2.27±0.27	2.92±0.08	2.78±0.15	3.00±0.00	2.88±0.13
Maxillary process	2.83±0.11	2.45±0.16	2.67±0.14	2.67±0.17	2.86±0.14	2.88±0.13
Mandibular process	2.00±0.00	1.73±0.14	2.00±0.00	1.89±0.11	2.00±0.00	2.00±0.00
Forelimb	2.00±0.00	1.55±0.16	1.83±0.11	1.56±0.18	1.29±0.18	1.25±0.16
Hindlimb	1.92±0.08	1.09±0.28*	1.83±0.11	1.56±0.18	1.00±0.00	1.13±0.13
Somites	5.00±0.00	4.82±0.12	5.00±0.00	4.89±0.11	5.00±0.00	5.00±0.00
Total score	61.38±0.75	53.83±2.47**	61.17±0.59	57.22±1.13**	60.29±0.52	60.63±0.82

Mann-Whitney U tests were used,

* P<0.05,

** P< 0.01,

data presented as Mean ± SEM.

Table 2

TUNEL staining quantification in B6 and 129S6 E8.2+2 embryos.

	B6		129S6	
	Control	**Alcohol**	**Control**	**Alcohol**
Forebrain	55.14±16.87	181.69±17.98 **	43.25±7.83	59.41±16.13
Midbrain	51.62±13.24	130.14±28.81 *	47.93±10.95	71.07±28.50
Hindbrain	106.26± 36.78	169.81±28.73	21.57±4.64	45.70±23.51
Cranial n. V.	65.53±4.03	118.98±34.28	11.48±3.46	38.37±27.30
VII–VIII	17.00±4.03	111.21±4.91 **	8.77±1.29	61.66±13.89 *
Branchial Arch	15.39±3.45	84.14±12.80 *	5.71±8.67	7.19±2.57
Heart	34.69±11.24	244.37±132.21	6.15±1.62	9.77±4.37
somites	64.85±10.50	369.70±72.34 *	95.70±38.35	88.56±22.36

* P<0.05 compare to control

** P<0.005 compare to control.

B6: n=4; 129S6:n=4

Data presented as Mean ± SEM.