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ABSTRACT

Genetic factors influence adverse pregnancy outcome in both humans and animal models. Animal research reveals both the maternal and fetal genetic profiles are important for determining the risk of physical birth defects and prenatal mortality. Using a reciprocal-cross breeding design, we investigated whether the mother's genes may be more important than fetal genes in determining risk for ethanol teratogenesis. Examination of possible synergistic genetic effects on ethanol teratogenesis was made possible by using two mouse strains known to be susceptible to specific malformations. Inbred A/J (A) and C57BL/6J (B6) mice were mated to produce four fetal genotype groups: the true-bred A·A and B6·B6 genotypes and the genetically identical A·B6 and B6·A genotypes (the F_1 genotype). Dams were administered either 5.8 g/kg ethanol or an isocaloric amount of maltose-dextrin on day 9 of pregnancy. Fetuses were removed by laparotomy on gestation day 18, weighed, and assessed for digit, vertebral, and kidney malformations. Digit malformations in the genetically identical F_1 ethanol-exposed litters showed a pattern consistent with a maternal genetic effect [A·B6 (2%) and B6·A (30%)]. In contrast, vertebral malformations were similar in all ethanol-exposed litters [A·A (26%), A·B6 (18%), B6·A (22%), and B6·B6 (33%)]. The percentage of malformations did not differ between male and female fetuses, indicating sex-linked factors are not responsible for the maternal effect. Ethanol exposure decreased litter weights but did not affect litter mortality compared to maltose-exposed controls. This study supports the idea that genes influence malformation risk following *in utero* alcohol exposure. Specifically, maternal genes influence risk more than fetal genes for some teratogenic outcomes. No evidence supported synergistic genetic effects on ethanol teratogenesis. This research supports the conclusion that uterine environment contributes to determining risk of Fetal Alcohol Spectrum Disorder.

Key words: Fetal Alcohol Syndrome; genetics; extra-chromosomal inheritance; inbred mice

INTRODUCTION

Women who drink alcohol while pregnant risk having children with congenital malformations. In the most extreme cases Fetal Alcohol Syndrome (FAS) may be diagnosed. The syndrome includes pre- and postnatal growth retardation, craniofacial abnormalities, and central nervous system dysfunction (Jones and Smith, 1973; Jones and Smith, 1975; Stratton et al., 1996). The designation Fetal Alcohol Spectrum Disorder or FASD is now used as an umbrella term to cover the range of outcomes associated with all levels of prenatal alcohol exposure (Hoyme et al., 2005; Sokol et al., 2003). Studies suggest rates of FAS and FASD in the range of 9-10/1000 live births (Lupton et al., 2004; May and Gossage, 2001; Sampson et al., 1997). The most devastating consequence to the individual is neurological damage leading to behavioral impairments and cognitive dysfunctions.

In humans there are a number of alcohol-related birth defects that include cardiac, skeletal, renal, and ocular malformations and dysplasias (Jones et al., 2010; Stratton et al., 1996). Though sporadic, malformations of these systems occur at a higher rate in children of mothers consuming alcohol than in the general population (see reviews by Burd et al., 2007; Hofer and Burd, 2009; Pauli and Feldman, 1986). Specific malformations include those involving digits (e.g., shortened digits, campodyactyly, clinodactyly, and radioulnar synostosis), kidney (e.g., aplastic, dysplastic, hypoplastic, and hydronephrosis), and vertebrae (vertebral fusions similar to the Klippel-Feil syndrome). However, not all women who consume ethanol during pregnancy give birth to FASD offspring. This indicates individual differences (both maternal and fetal) in ethanol teratogenesis susceptibility. Many risk factors play a role in FASD development (Abel and Hannigan, 1995; May et al., 2008) and several studies point to genetic differences in susceptibility (reviewed by Becker et al., 1996; Gemma et al., 2006; Warren and Li, 2005).

Mice are useful in studying the mechanisms of FASD. All hallmark features of FASD can be replicated in mice (Driscoll et al., 1990) including prenatal and postnatal growth retardation, morphological malformations, and central nervous system dysfunction, which includes behavioral abnormalities. Experiments using inbred and selectively bred mouse stocks that controlled for 1) ethanol dose, 2) maternal and fetal blood ethanol levels, and 3) fetal developmental exposure stage, show that genotype can affect teratogenic outcome. The deleterious outcomes under genetic control include differing levels of embryo lethality, brain morphology, fetal weight gain, and digit, skeletal, ocular, renal and heart anomalies, as well as behavioral anomalies (Boehm et al., 1997; Gilliam and Kotch,

1990; Gilliam and Kotch, 1992; Gilliam and Kotch, 1996; Gilliam et al., 1989; Gilliam et al., 1987; Goodlett et al., 1989).

Other experiments helped distinguish the teratogenic effects mediated by maternal genotype from those mediated by fetal genotype (Downing and Gilliam, 1999; Gilliam and Irtenkauf, 1990; Gilliam et al., 1997). Reciprocal breeding between distinct mouse stocks accomplishes this. A *maternal effect* is indicated when genetically identical F_1 offspring of the reciprocal heterozygotes differ in teratogenic response (Biddle and Fraser, 1977). If the difference is limited to males or found at a higher rate in males, the maternal effect may be attributed to X-linked genes. When the difference is not male-specific, maternal effects are presumably due to cytoplasmic inheritance, maternal physiology affecting the uterine environment, or epigenetic phenomena.

Each previous study examining maternal effects on ethanol teratogenesis employed reciprocal crosses between a susceptible mouse genotype (the C57BL/6J or B6 mouse) and a *relatively resistant* mouse genotype (*e.g.*, Long-Sleep, Short-Sleep, or DBA/2J mice). In each instance F_1 offspring with a susceptible B6 mother exhibited significantly greater teratogenic effects than genetically identical F_1 offspring with a relatively resistant mother. In a reciprocal cross between B6 and selectively-bred Long-Sleep (LS) mice, the effect of the B6 mother on F_1 litters was to increase fetal weight deficits, kidney malformations, and digit malformations compared to the effect of LS mothers (Gilliam and Irtenkauf, 1990). In a cross between B6 and selectively-bred Short-Sleep (SS) mice ethanol-exposed B6 mothers increased digit malformations in F_1 litters compared to similarly treated SS mothers (Gilliam et al., 1997). Finally, in an experiment designed to examine both maternal genetic effects and whether cytoplasmic factors were responsible, rib and vertebral malformations were increased in ethanol treated F_1 litters from B6 mothers but not in F_1 litters from DBA/2J (D2) mothers. Importantly, the source of the maternal effect could not be ascribed to factors transmitted through the egg cytoplasm or sex-linked genes (Downing and Gilliam, 1999). In each of these experiments the B6 mother increased ethanol teratogenic severity compared to mothers from less affected mouse stocks. Furthermore, blood ethanol levels were similar among ethanol-exposed groups regardless of mouse stock.

Maternal effects on ethanol teratogenesis have not been examined between two susceptible mouse stocks. The possibility of synergistic genetic effects - when the joint contribution of parental alleles is greater than the sum of their individual effects (see Perez-Perez et al., 2009) - remains to be examined. There are very few ethanol

teratology studies where different genotypes were compared side-by-side. In one study where three genotypes were compared, Boehm (Boehm et al., 1997) found both C57BL/6J and A/J mice to show significantly increased vertebral malformations in ethanol-exposed compared to maltose-exposed litters. Importantly, the treatment effect, *i.e.*, the difference between ethanol- and maltose-exposed litters, was greater in A/J than C57BL/6J mice. An examination of vertebral malformations in the F_1 offspring of these strains may reveal synergistic genetic effects not seen in previous mating designs.

The purpose of this experiment was: (1) to confirm previously reported teratogenic effects of ethanol in two sensitive inbred mouse strains (the A/J or A and C57BL/6J or B6 mice) using standard treatment procedures and assessment techniques, (2) to examine the role of maternal versus fetal genetic susceptibility in teratogenic etiology, and (3) to examine the potential of synergistic genetic effects on ethanol teratology. To accomplish this, we compared the teratogenic effects of ethanol in true-bred and reciprocally-bred A and B6 litters.

MATERIALS AND METHODS

Subjects

Experimental animals were A/J (A) and C57BL/6J (B6) mice. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at five weeks of age. Male and nulliparous females were housed by sex in OptiMICE® cages, each holding three to five animals. A 12-hr light cycle (0700 to 1900 hr) was maintained. Food (Purina 5015 High Protein Mouse Chow) and water were provided *ad libitum*. Room temperature was maintained at 20º to 22ºC. At 70 days of age males were housed singly. Pairs of female mice were placed with a male after meeting a minimum 17-g weight criterion. In order to constrain the time of conception and thus the phase of embryonic development relative to the time of exposure to ethanol, A or B6 females were place with either an A or B6 male for 3 hr (0900 to 1200 hr). At the end of the three-hour period, females were examined for a vaginal plug as evidence of mating. Females showing a vaginal plug were weighed and single-housed. The day of plug detection was designated as day 0 of pregnancy. Females were randomly assigned to treatment groups: either an ethanol group or a maltose-dextrin control group within each of the four fetal genotype groups. A non-intubated control group was not included in the experimental design because preliminary studies showed no differences in the frequency of malformations between non-intubated groups and groups intubated with maltose-dextrin for either A or

B6 mice (unpublished observations; see also Boehm et al., 1997). Four fetal genotype groups were formed by this mating procedure: true-bred A·A and B6·B6 genotypes, and hybrid A·B6 and B6·A genotypes. (Note: fetal genotypes are written with the maternal genotype first). The reciprocally bred A·B6 and B6·A fetuses are genetically identical with the following exception: the male fetuses differ in origin of their sex chromosomes. The A·B6 male fetuses receive their X chromosome from their A mother and their Y chromosome from their B6 father, whereas the B6·A fetuses receive their X chromosome from their B6 mother and their Y chromosome from their A father.

The research protocol (#0703) was reviewed in advance by the University of Northern Colorado Institutional Animal Care and Use Committee and was conducted according to the requirements of all local, national, and international standards for the care and use of laboratory animals (the specific guidelines followed were the NIH Guide for the Care and Use of Laboratory Animals (PHS, 1996).

Dosing Regimen

On day 9 of pregnancy, females were weighed between 1200 and 1300 hr and determined to be pregnant by meeting a minimum 2-g weight-gain criterion based on their day 0 weights. Between 1230 and 1330 hr, pregnant females were intragastrically intubated with either 5.8 g/kg ethanol (20% w/v) or an isocaloric amount of a maltose-dextrin solution (35% w/v). This dose was used because it was previously shown to result in similar blood ethanol levels (439 mg/dl at 60 min after intubation) and significantly increased vertebral malformations in both B6 and A mice (Boehm et al., 1997). Blood ethanol levels were not measured in this study. Mouse embryonic development on day 9 is similar to human embryonic development at the end of the first month of pregnancy. Since this ethanol dose produced increased vertebral malformations in both true-bred A and B6 litters, it allowed us to examine synergistic genetic effects - whether reciprocal F_1 litters showed higher frequencies of vertebral malformations than true-bred litters. In contrast with previous studies, no food restriction was implemented in the present study. We have found that mice in our laboratory eat food during the lights-off period (six hours before and after intubation). We felt it unnecessary to restrict food prior to or after intubations because of this. Females were again weighed between 1200 and 1300 hr on day 10 of pregnancy.

Fetal Examination

On day 18 of pregnancy, females were weighed, killed by $C0_2$ inhalation, and necropsied between 1500 and 1700 hr. Uterine horns were exposed and a count made of live and resorbed fetuses (no dead fetuses were observed). Early and late resorptions were differentiated as defined by Gleich and Frohberg (1977). Live fetuses were sexed, weighed (rounded to 0.001 g), and examined for gross external morphological defects (eye, cleft-lip & palate, front and hind digits and limbs, head and snout shape, limb flexion, tail, and torso). Every other fetus within a litter was placed in Bouin's fixative a minimum of two weeks for subsequent soft tissue analysis using Wilson's (1965) freehand slicing method. The remaining fetuses were eviscerated and stored in ethanol a minimum of two weeks for subsequent skeletal evaluation (we routinely use the Sterling-Winthrop Research Institute Manual for Skeletal Evaluation). Fetal examinations were done without knowledge of maternal condition. A 1200 hr necropsy time was necessitated for all B6·A litters because several maltose-exposed litters were born by 1300 hr on day 18 of pregnancy.

Statistical Analysis

Power Analysis: Previous experiments show B6 litters are sensitive to weight deficits, as well as digit, skeletal (vertebral and rib), and soft-tissue (kidney) malformations following *in utero* exposure to a 5.8 g/kg dose on day 9 of pregnancy (Boehm et al., 1997; Downing and Gilliam, 1999; Gilliam and Irtenkauf, 1990; Gilliam et al., 1997). In a side-by-side comparison with B6 litters, A litters were shown to be sensitive to weight deficits and vertebral malformations, but not digit and kidney malformations following exposure on day 9 of gestation (Boehm et al., 1997). Using historical data, power analysis was performed to determine the minimum number of litters needed to find a difference in percentage digit malformations between the A and B6 genotypes. Sample values used were: mean difference = 40 and standard deviation = 25. Analysis, using SAS® statistics, showed that the minimum *n* need for a power of 0.7 is 14 (7 litters/genotype). This indicates that in order to have an adequate power of 0.7 or greater, a minimum of 7 litters per genotype is required (14 total). Increasing the number of litters to 10 per genotype, achieves an excellent power of 0.9.

Maternal and litter data were examined using analysis of variance (ANOVA) with fetal genotype (A·A, A·B6, B6·A, and B6·B6) and treatment (ethanol and maltose-dextrin) as between-group factors. Fetal

malformations (digit and vertebral) and fetal weight were examined with multivariate ANOVAs. The mean litter percentages malformed for each sex, or mean litter weight for each sex, were used as units of analyses. Other malformations (kidney, cleft lip, and eye) occurred at very low frequencies; whole litter mean percentages were used as the unit of analysis in univariate ANOVAs. Litters with only one implantation were not used in any analysis. Because percentage means and variances tend to be related, percentage scores of maternal weight gain, fetal mortality, and malformations were transformed {2[arcsin(percent$^{.5}$)]} prior to analysis, as recommended by Winer (1991). Treatment contrasts of reciprocally-bred genotypes (A·B6 and B6·A) were performed to examine the presence of a maternal effect. Other contrasts were performed to compare the average true-bred value (A·A, B6·B6) to the average reciprocally-bred value (A·B6, B6·A). This contrast assesses the presence of genetic dominance. Further analyses used simple effect and post-hoc comparisons (Newman-Keuls), where appropriate, after significant (p<0.05) results. Reported values are mean and standard error of the mean. Eta squared values were calculated by $SS_{effect}/SS_{corrected\ total}$; partial eta squared values were taken from the SPSS 16.0 output.

RESULTS

Maternal Weight

B6 dams gained more weight than A dams regardless of fetal genotype (Table 1). Ethanol treatment reduced percent maternal weight gain [$F(1,86)=4.73$, $p<.05$; $\acute{\eta}^2=.03$] regardless of maternal genotype. However, when maternal weight gain was adjusted for litter weight (litter weight subtracted out), the treatment effect disappeared [$F(1,86)=0.53$, $p=.47$]. This indicates maternal weight gain was less for ethanol-exposed compared to maltose-exposed litters only because litters differed in weight. Genotype also influenced maternal weight gain [$F(3,86)=20.17$, $p<.05$; $\acute{\eta}^2=.40$] in spite of litter weight adjustments [$F(3,86)=13.39$, $p<.05$; $\acute{\eta}^2=.32$]. A·A dams gained less weight than A·B6 dams, which gained less than either B6·A or B6·B6 dams (p's<.05). Four dams had total resorptions (one B6·A maltose, one A·B6 maltose, and two A·A ethanol) and were not included in the analysis of maternal weight gain.

Implantation Sites

Implantation sites can be accurately measured on totally resorbed litters. Implants are expected to be relatively consistent within inbred genotypes and independent of pregnancy day-9 treatment. In this experiment the

number of sites ranged from six to 10 for B6 dams, and four to 12 for A dams. The largest mean difference between treatment groups for a genotype was 1.3 implantation sites observed in the A·A dams (Table 1). Analysis revealed a genotype by treatment interaction on implantation sites [$F(3,90)=4.01$, $p<.05$; $\eta^2=.11$]. Treatment contrasts showed the interaction occurred between the A·A and A·B6 genotypes: the A·A maltose group had significantly more sites than the A·A ethanol group, while the A·B6 ethanol group had significantly more sites than the A·B6 maltose group (p's<.05). An A·A vs. A·B6 comparison, averaged across treatments, showed no significant difference ($t(48)=.298$, $p=.77$), which suggests the genotype by treatment effect was due to a chance distribution of A dams within treatment groups with a large or small number of implants. Implantation sites did not differ between treatment groups for B6 dams (*i.e.*, B6·A and B6·B6 litters).

Prenatal Mortality

Fetal mortality, measured as a percentage of resorptions per number of implants, was greatest in the A·A ethanol group and averaged 47%. All other treatment group by genotype combinations averaged less than 25% (Table 1). Analysis showed only a genotype effect on fetal mortality [($F(3,90)=5.65$, $p<.05$; $\eta^2=.15$]. The A·A genotype had significantly more fetal mortality than all other genotypes (p's<.05).

Fetal Weight

As expected, hybrid litters weighed more than true-bred litters and ethanol-treated litters weighed less than maltose-treated litters (E mean = 1.04929 g ± .02502 g vs. MD mean = 1.07788 g ± .02877 g; Table 1). Results of the multivariate analysis of male and female weights showed significant differences for genotype [Roy's largest root $F(3,82)=71.612$, $p<.05$; partial $\eta^2=.72$] and treatment [Roy's largest root $F(2,81)=4.386$, $p<.05$; partial $\eta^2=.10$], but no interaction. A·A litters weighed less than A·B6 and B6·B6 litters, while all three weighed less than B6·A litters (p's<.05). All univariate analyses and post-hoc tests (on genotype) revealed identical results, whether male or female weights were examined. Male and female weights within a litter were highly correlated ($r = .90$, $p<.05$), with males (1.07701 g ± 0.02001 g) weighing more than females (1.05171 g ± 0.01999 g; *paired* $t(89)=2.855$, $p<.05$).

Malformations

Digit malformations were tallied on all live fetuses at the time of laparotomy. Malformations consisted of fused or missing digits on either forepaw (Table 2). A small number of digit malformations were only discovered at the time of skeletal examination; some were accompanied by a missing or foreshortened ulna). The majority of digit malformations were observed in ethanol-exposed B6·A and B6·B6 litters. Analysis of male and female litter percentages showed a genotype by treatment interaction [Roy's largest root $F(3,82)=3.38$, $p<.05$; partial $\acute{\eta}^2=.11$]. For each fetal genotype, males and females had similar percentages of digit malformations within each treatment group (data not shown). Ethanol exposure increased digit malformations in B6·B6 and B6·A litters (p's$<.05$) compared to maltose controls. In contrast, there were no treatment effect differences in either A·A or A·B6 litters. A maternal effect was indicated by finding that ethanol-exposed B6·A litters had more digit malformations than ethanol-exposed A·B6 litters ($p<.05$; Figure 1). The absence of genetic dominance was shown by finding no difference in the average true-bred value (A·A, B6·B6) compared to the average hybrid value (A·B6, B6·A). Finally, litters with a B6 mother (B6·B6 and B6·A) had more digit malformations than litters with an A mother (A·A and A·B6; $p<.05$).

Cleft lip was restricted to the A·A genotype and unaffected by treatment (Table 2). Analysis showed only a genotype effect [$F(3,86)=8.10$, $p<.05$; $\acute{\eta}^2=.22$]; the A·A genotype exhibited a higher percentage of cleft lip than all other genotypes ($p's<.05$). Eye malformations included either small or missing eye but were infrequent (Table 2). Analysis revealed no effects on averaged litter mean percentages.

Kidney malformations (hydronephrosis) were assessed on every other fetus within each litter using Wilson's free-hand sectioning technique (Wilson, 1965). While occurring at low levels for most genotype by treatment combinations, kidney malformations were greatest among ethanol-treated B6·B6 litters (Table 2). Analysis showed a genotype by treatment interaction [$F(3,84)=2.68$, $p<.05$; $\acute{\eta}^2=.09$]. Simple-effect analysis indicated a higher percentage of kidney malformations among ethanol-exposed than maltose-exposed B6·B6 litters ($p<.05$). No other treatment comparisons were significant.

Skeletal malformations consisted of fused or asymmetrical vertebral arches and/or centra. Rib malformations averaged less than 1% for any genotype by treatment group and were included in vertebral malformation percentages. In general, ethanol increased the percentage of vertebral malformations regardless of fetal genotype (Table 2). Analysis of male and female litter percentages revealed only a significant treatment effect

[Roy's largest root $F(2,73)=8.48$, $p<.05$; partial $\eta^2=.19$; E > MD]. Within each genotype by treatment group, males and females showed similar mean percentages of vertebral malformations (data not shown). The A and B6 genotypes appear to represent a common susceptibility category to vertebral malformations following *in utero* ethanol exposure.

Within a given genotype, the timing of ethanol exposure is known to produce different teratogenic outcomes (see Discussion). To shed light on this phenomenon, we examined the relationship between digit malformations, where we found a maternal effect (B6·A > A·B6), and vertebral malformations, which showed elevated percentages in all ethanol-exposed groups. Analysis showed fetuses within the same litter have a greater chance of digit malformations if vertebral malformations are also seen in other fetuses from that litter [$\chi^2(1)=20.76$, $p<.001$; Table 3]. This finding indicates a greater chance of having both malformations rather than one malformation within the same litter. Furthermore, it indicates the maternal effect on digit malformations is not due to developmental timing differences between the A·B6 and B6·A genotypes.

DISCUSSION

In this study we confirmed previously reported ethanol teratogenic effects in the A/J and C57BL/6J mouse strains (Boehm et al., 1997). B6 mice show ethanol teratogenic effects across several domains (digit, kidney, and vertebrae), while A mice show effects on vertebrae, although not to the extent previously reported (Boehm et al., 1997). We found prenatal alcohol exposure in reciprocally-bred fetuses produced differing percentages of digit malformations. F_1 fetuses carried in a B6 mother had a higher rate of digit malformations than genetically identical F_1 fetuses carried in an A mother. This result indicates B6 maternal genes contribute significantly to a risk for ethanol teratogenesis, while A maternal genes reduce risk. Furthermore, digit and vertebral malformations both occurred within the same litter, indicating malformations were produced at the same critical developmental stage. In agreement with other studies (Downing and Gilliam, 1999; Gilliam and Irtenkauf, 1990; Gilliam et al., 1997), B6 mothers consistently have offspring more susceptible to ethanol teratogenesis, regardless of fetal genotype. The unique malformations seen in specific reciprocal F_1 genotypes with a B6 mother may result from either uterine physiology affecting fetal development, ethanol's direct effect on maternally or paternally expressed genes in the fetus, or unique parental cytoplasmic contributions to the gamete. The latter possibility seems unlikely because all

common inbred mouse strains share the same mitochondrial DNA restriction pattern of *M. musculus domesticus* (Carlier et al., 1992), the major genetic component of maternal egg cytoplasm.

B6 and A mice are two widely used inbred strains in biomedical research and exhibit many phenotype differences (Marshall et al., 1992; Nesbitt et al., 1979). However, in regards to genetic analyses, they represent only two genotypes and analysis of additional strains is warranted in order to characterize the range of genetic variation for ethanol teratogenesis (see Downing et al., 2009). While demonstrating inbred strain differences shows that genetics can influence a phenotype, the ultimate goal of most genetic research is to identify the genes and DNA polymorphisms mediating differential sensitivity to a phenotype. Recombinant inbred (RI) strains are populations of mice used to identify quantitative trait loci (QTL), regions on chromosomes that harbor genes or DNA polymorphisms that mediate differential sensitivity to a phenotype. One potentially useful mapping panel is the AXB/BXA series of RI strains. The AXB/BXA panel was created by crossing B6 females with A males and by crossing A females with B6 males. Thus, comparison of AXB strains with BXA strains will allow researchers to further investigate and/or verify maternal effects. The largest available panel of RIs, the BXD RIs, was created by crossing C57BL/6J (B6) females with DBA/2J (D2) males. Over 80 BXD RI strains are available, and are currently being used to map QTLs mediating ethanol teratogenic effects.

An important question is whether or not fetuses from diverse mouse stocks are at the same embryonic development stage when exposed to a teratogen. A measure often used to quantify stage of embryological development is somite number. As in other vertebrate embryos, the mouse embryo undergoes tissue segmentation as seen by somite formation during organogenesis (Gossler and Tam, 2002). In general, about 65 somite pairs are progressively formed in a head-to-tail direction beginning on embryonic day 8 and ending on embryonic day 13.5 (Kaufman and Bard, 1999). Somite number at a specific gestational time point can vary greatly. This is true not only among genetically identical embryos within the same litter, but also between litters of the same inbred mouse strain (Thiel et al., 1993). In other words, embryonic development during organogenesis does not proceed in parallel for genetically identical embryos within or between litters. A 3 to 8 somite variation among mouse embryos from the same litter may represent a 5 to 16 hour difference of development (Tam, 1981). An ideal experimental procedure would determine, for example, both somite number at the exact time of ethanol exposure *and* day-18 teratogenic endpoint on the *same* mouse pup. This would link developmental stage and malformation. However, at

present, this is technically impossible. Although fetuses within the same B6 or A litter in this study are most likely at different developmental stages, other factors (see below) may be more important in determining damage due to ethanol exposure.

One method to limit developmental-stage differences among embryos is to use embryo-culture techniques. In a well-controlled experiment, Ogawa et al. (2005) investigated the effect of ethanol exposure on day-8, cultured whole embryos with 3 to 6 somites from two strains. This experiment employed identical ethanol-exposure concentrations, patterns, and durations. B6 and D2 inbred mouse embryos were saturated with ethanol for 44 hours; culture medium ethanol concentrations ranged from 175 to 440 mg/dl during the entire exposure period. Interestingly, even when somite number was well controlled between strains, strain specific embryonic regions showed different susceptibilities to developmental delay when assessed after 44 hours. In B6 embryos, the heart, caudal neural tube, forebrain, optic system, and hind limb were significantly compromised by ethanol exposure compared to controls. For D2 embryos the hindbrain, forebrain, optic system, bronchial bars, and forelimb were significantly compromised by ethanol exposure compared to controls (Ogawa et al., 2005). When we exposed D2 litters to 5.8 g/kg ethanol on day 9 or day 10 of pregnancy, we did not find significantly elevated malformation percentages. Although the embryo-culture technique allows very precise control over experimental variables, maternally-mediated factors such as uterine environment may play a significant role in teratogenic susceptibility and resistance.

Ethanol-induced limb malformations in B6 fetuses involve cell death in the developing apical ectodermal ridge (Kotch et al., 1992) and probable interference with retinoic acid gene signaling and gene products (Johnson et al., 2003; Johnson et al., 2007). Fetal B6 limb bud cell death and malformations were reduced by administering antioxidents to B6 mothers (Chen et al., 2004). Differences in reciprocal heterozygote digit malformations seen in this study may be due to maternal differences in reactive oxygen-scavenging capacities. B6 dams may produce more reactive oxygen species (ROS) than A dams or have less capacity to cope with high ROS levels. The end result of oxidant/anti-oxidant interference systems may lie in formation of secondary free radicals formed from scavenger systems (Ohtake et al., 1997). In addition, ROS may alter gene expression (Turpaev, 2002). High versus low maternal ROS levels may differentially affect fetal gene expression even among genetically identical F_1 litters.

Future experiments should explore how maternal genotype impacts fetal risk for prenatal alcohol effects (see Hager et al., 2008, for a discussion of possible strategies).

Other research has also implicated maternal genotype (rather than fetal genotype) in prenatal alcohol effects. In an experiment comparing parental mice with either increased or decreased superoxide dismutase (SOD) activity, Wentzel and Eriksson (2006) found that enhanced maternal antioxidative capacity reduced ethanol teratogenesis. Moreover, maternal genotype influenced pregnancy outcome more than fetal genotype. They suggested that maternal oxidative stress may promote transfer of teratogenic substances (like isoprostanes) to fetuses. Excellent reviews are available of antioxidants and fetal protection from alcohol (Cohen-Kerem and Koren, 2003), as well as other mechanisms of fetal damage (Goodlett et al., 2005).

The advantage of comparing inbred strains – and, more specifically, comparing genetically identical offspring (i.e., F_1's) gestating within differing, but genetically and experimentally consistent maternal uterine environments, – is of course vastly different than what occurs in the human condition. Inbred strains represent unique genetic combinations that are homozygous at all chromosomal loci. They are by their very nature more labile to any environmental insult/change because of their lack of genetic diversity – that is they lack genetic buffering (Waddington, 1942) – the compensatory process whereby particular gene activities confer phenotypic stability against genetic or environmental variations (Hartman IV, 2006). The unique genetic combinations that promote ethanol teratogenic risk in A and B6 mice may never occur in humans. But what this and our other studies do suggest is that there is interplay between those genes increasing fetal risk and the uterine environment as modulated by maternal genetics.

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Figure 1. Percent digit malformations in ethanol-exposed true-bred (P_1 and P_2) and reciprocally bred (F_1) fetal genotypes. Fetal genotypes are shown as parental female x parental male. A = A/J mice and B6 = C57BL/6J mice. The greater percentage of malformations in the B6·A group compared with the A·B6 group ($p < 0.05$) is attributed to a difference in maternal response to ethanol (maternal effect).

Table 1. Mean (±SEM) % Maternal Weight Gain, Adjusted % Maternal Weight Gain, Implantation Sites, Litter Mortality, and Fetal Weights

	A·A		A·B6		B6·A		B6·B6	
	Maltose	Ethanol	Maltose	Ethanol	Maltose	Ethanol	Maltose	Ethanol
Maternal Data								
No. of litters[a]	13	9	11	13	10	12	13	12
% Weight gain[b,c]	56.3 (±2.3)	47.5 (±3.6)	71.9 (±4.9)	70.8 (±4.5)	89.5 (±5.6)	82.4 (±4.3)	82.6 (±4.5)	73.5 (±5.0)
Adjusted % weight gain[d,e]	27.4 (±1.9)	26.1 (±2.0)	31.9 (±2.3)	30.7 (±1.9)	39.6 (±2.2)	37.0 (±2.3)	37.6 (±2.3)	38.3 (±1.9)
No. Implants per litter[f]	9.38 (±.46)	8.08 (±.50)	8.25 (±.41)	9.54 (±.64)	7.73 (±.43)	8.25 (±.39)	8.69 (±.21)	7.75 (±.25)
Fetal Data								
% Litter mortality[g]	24.2 (±3.9)	47.3 (±9.7)	21.6 (±8.9)	20.9 (±3.4)	17.7 (±9.5)	13.9 (±2.9)	9.8 (±4.1)	16.9 (±7.7)
Weight[h] (g): Males	0.81616 (±.02062)	0.84530 (±.02214)	1.12988 (±.04133)	1.06882 (±.02437)	1.30268 (±.04740)	1.22168 (±.04293)	1.14835 (±.01807)	1.07163 (±.03677)
Weight (g): Females	0.82823 (±.01986)	0.76041 (±.02714)	1.0707 (±.03446)	1.04095 (±.01949)	1.27986 (±.04546)	1.20183 (±.04734)	1.13897 (±.01386)	1.07606 (±.03297)

[a] One A·A ethanol litter had only females and one had only males; two B6·B6 ethanol litters had only females.

[b] % weight gain = [(Day 18 weight – Day 0 weight) ÷ (Day 0 weight)] x 100

[c] A·A < A·B6 < B6·B6; *p*'s < .05; A·B6 = B6·A

[d] Adjusted % weight = {[(Day 18 weight – Day 0 weight) – total litter weight] ÷ Day 0 weight} x 100

[e] A·A < A·B6 < B6·A = B6·B6; *p*'s < .05

[f] A·A Maltose > A·A Ethanol; A·B6 Maltose < A·B6 Ethanol (*p*'s < .05)

[g] A·A > B6·A and B6·B6 (*p*'s < .05).

[h] (Male & Female): A·A < A·B6 = B6·B6 < B6·A; Ethanol < Maltose

Table 2. Malformations: Mean (±SEM) percentage of litter means

	A·A		A·B6		B6·A		B6·B6	
	Maltose	Ethanol	Maltose	Ethanol	Maltose	Ethanol	Maltose	Ethanol
Gross malformations								
Digit[a]	0	5.0 (±5.00) **[1/53][e]**	0	2.2 (±2.20) **[2/99]**	0	29.6 (±11.4) **[28/86]**	0	20.4 (±10.2) **[17/76]**
Cleft lip[b]	6.8 (±3.2) **[6/94]**	3.4 (±2.3) **[2/53]**	0	0	0	0	0	0
Eye	2.4 (±1.7) **[2/94]**	2.0 (±2.0) **[1/53]**	0	0	0	0.9 (±0.9) **[1/86]**	2.5 (±1.7) **[2/102]**	0
Soft tissue malformations								
Kidney[c]	5.1 (±3.5) **[2/47]**	0	6.1 (±6.1) **[2/40]**	7.3 (±4.3) **[3/49]**	5.0 (±5.0) **[1/38]**	8.3 (±8.3) **[3/43]**	1.5 (±1.5) **[1/49]**	26.1 (±9.1) **[10/38]**
Skeletal malformations								
Vertebral arch & centra[d]	9.0 (±4.9) **[3/47]**	25.8 (±9.5) **[8/26]**	2.3 (±2.3) **[1/37]**	17.9 (±10.4) **[6/50]**	0	21.5 (±9.0) **[10/44]**	0	33.3 (±11.0) **[14/38]**

[a] B6·A and B6·B6: Ethanol >Maltose, *p*'s<.05

[b] A·A > A·B6 = B6·A = B6·B6, *p*'s<.05

[c] B6·B6: Ethanol > Maltose, *p*<.05

[d] Ethanol > Maltose; *p*<.05

[e] [total number with malformation / total number examined]

Table 3. Litters with any digit and / or skeletal malformations

		Litters with digit malformations		
		0	1	Total
Litters with vertebral malformations	0	68	2	70
	1	15	9	24
	Total	83	11	94

0 = absence of malformation
1= presence of malformation
$\chi^2(1)=20.758$, $p<.001$

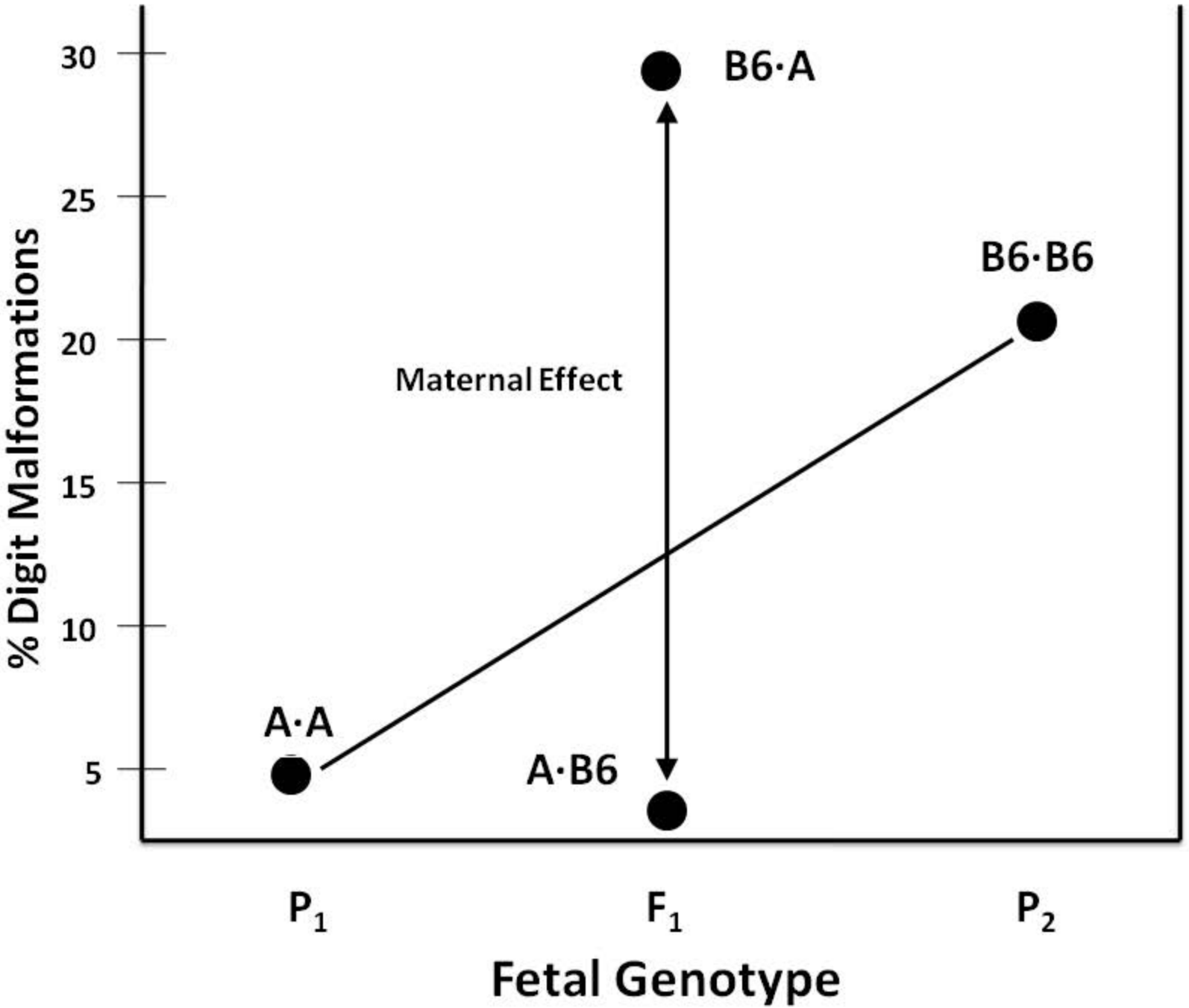
30
25
20
15
10
5
% Digit Malformations
B6·A
B6·B6
Maternal Effect
A·A
A·B6
P1
F1
P2
Fetal Genotype

Figure 1. Percent digit malformations in ethanol-exposed true-bred (P_1 and P_2) and reciprocally bred (F_1) fetal genotypes. Fetal genotypes are shown as parental female x parental male. A = A/J mice and B6 = C57BL/6J mice. The greater percentage of malformations in the B6·A group compared with the A·B6 group ($p < 0.05$) is attributed to a difference in maternal response to ethanol (maternal effect).