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Methanol Exposure During Gastrulation Causes Holoprosencephaly, Facial Dysgenesis, and Cervical Vertebral Malformations in C57BL/6J Mice

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BACKGROUND: Exposure of pregnant outbred CD-1 mice to methanol during the period of gastrulation results in exencephaly, cleft palate, and cervical vertebra malformations [Rogers and Mole, Teratology 55: 364, 1997], while inbred C57BL/6J mice are sensitive to the teratogenicity of ethanol. C57BL/6J fetuses exhibit the holoprosencephaly spectrum of malformations after maternal exposure to ethanol during gastrulation, but the sensitivity of C57BL/6J mice to methanol-induced teratogenesis has not been previously described. **METHODS:** Pregnant C57BL/6J mice were administered two i.p. injections totaling 3.4 or 4.9 g/kg methanol or distilled water four hrs apart on gestation day (GD) 7. On GD 17, litters were examined for numbers of live, dead and resorbed conceptuses, fetuses were weighed as a litter and examined externally, and all fetuses were double stained for skeletal analysis. **RESULTS:** No maternal intoxication was apparent, but the high dosage level caused a transient deficit in maternal weight gain. The number of live fetuses per litter was reduced at both dosages of methanol, and fetal weight was lower in the high dosage group. Craniofacial defects were observed in 55.8% of fetuses in the low dosage group and 91.0% of fetuses in the high dosage group, including micro/anophthalmia, holoprosencephaly, facial clefts and gross facial angenesis. Skeletal malformations, particularly of the cervical vertebrae, were observed at both dosages of methanol, and were similar to those previously reported in the CD-1 mouse following methanol exposure. **CONCLUSIONS:** The types of craniofacial malformations induced in the C57BL/6J mouse by methanol indicate that methanol and ethanol have common targets and may have common modes of action. *Birth Defects Res B* 71:80–88, 2004. Published 2004 Wiley-Liss, Inc. †

INTRODUCTION

Methanol (MeOH) is used in the production of methyl tert-butyl ether (MTBE), formaldehyde, acetic acid, and methyl methacrylate, and is widely used as an industrial and laboratory solvent. The U.S. Environmental Protection Agency (1994) reported that MeOH was the chemical with highest release to the environment (air, water, and land) based on the 1992 Toxic Release Inventory of 23,630 facilities. Releases of MeOH totaled more than 214 million pounds. It is a component of a number of widely used consumer products including antifreeze, windshield washer fluids, solvents for duplicating machines and paint (Conibear, 1988). MeOH has also been used as an automobile fuel in gasoline blends or as pure MeOH. Although much is known about the acute toxicity of MeOH in humans (Kavet and Nauss, 1990), developmental toxicity in humans has not been described.

The developmental toxicity of MeOH has been studied in rats and mice. Nelson et al. (1985) have shown that high doses of inhaled MeOH (10,000–20,000 ppm, 7 hr/day on gestation day [GD] 1–19 or 7–15) result in maternal and developmental toxicity in rats. Altered behavioral development of offspring after exposure of pregnant Long–Evans rats to 2% MeOH in the drinking water has also been reported (Infurna and Weiss, 1986). Mice are more sensitive to the developmental toxicity of inhaled MeOH than are rats. We have reported cleft palate, exencephaly, and skeletal malformations in fetuses of pregnant outbred CD-1 mice exposed to 5,000 ppm MeOH or greater for 7 hr/day on GD 6–15, and an increased incidence of ribs lateral to the seventh cervical vertebra in fetuses of dams exposed to 2,000 ppm during the same periods (Rogers et al., 1993). The "no observed adverse effect level" (NOAEL) in that

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study was 1,000 ppm. Methanol oxidation occurs in part through a folate-dependent pathway, and folate deficiency has been shown to exacerbate some aspects of the developmental toxicity of methanol in CD-1 mice (Fu et al., 1996; Sakanashi et al., 1996).

The greater sensitivity of the CD-1 mouse compared to the Sprague–Dawley rat probably involves species differences in both maternal uptake of MeOH by inhalation and intrinsic differences in embryo sensitivity. Comparison of blood MeOH concentrations attained after similar inhalation exposures indicates that peak blood MeOH concentrations are approximately twice as high in mice (Rogers et al., 1993) as in rats (Nelson et al., 1985). Exposure of mouse and rat embryos to MeOH in whole embryo culture demonstrated that maternal metabolism of MeOH is not required for developmental toxicity and that mouse embryos are intrinsically more sensitive to MeOH than are rat embryos (Andrews et al., 1993).

Bolon et al. (1993) reported embryo lethality, exencephaly, cleft palate, and digit defects among litters of CD-1 mouse dams exposed to 10,000 ppm MeOH. These investigators also reported that GD 7–8 or 8–9 were the most sensitive for induction of neural tube defects by 2-day exposures to 15,000 ppm MeOH for 6 hr/day. Cleft palate was induced by maternal MeOH exposure to 10,000 ppm on GD 6–15 or GD 7–9, or by exposure to 15,000 ppm on GD 9–11. Fetal skeletons were not evaluated in these studies. We have shown that GD 7 (gastrulation) is the most sensitive period for induction of malformations of cervical vertebra in CD-1 mice, and these defects include homeotic transformations of the cervical vertebra (Connelly and Rogers, 1997; Rogers and Mole, 1997).

The C57BL/6J mouse strain has been used extensively as a model for the study of the pathogenesis of fetal alcohol syndrome and alcohol-related birth defects in humans (Sulik et al., 1981, 1984; Sulik and Johnson, 1983; Webster et al., 1983, 1984; Cook et al., 1987; Webster and Ritchie, 1991; Kotch and Sulik, 1995; Dunty et al., 2001, 2002). These studies have demonstrated that gastrulation stage ethanol exposure of pregnant C57BL/6J mice results in severe craniofacial malformations including micro/anophthalmia, facial clefts, facial dysgenesis and holoprosencephaly. Because of the extensive literature base on the effects of ethanol on craniofacial development in the C57BL/6J mouse, we evaluated the effects of gastrulation-stage exposure to methanol on development of the C57BL/6J mouse embryo. Using this mouse strain to compare the developmental effects of methanol to the well-documented effects of the human teratogen, ethanol, should provide evidence on whether these two alcohols may have similar targets. Our results indicate that, in C57BL/6J mice, methanol causes craniofacial malformations indistinguishable from those caused by ethanol, suggesting that these two alcohols may work though similar modes of action when administered during gastrulation.

MATERIALS AND METHODS

Animals and Treatment

Sixty day old nulliparous female C57BL/6J mice obtained from Jackson Laboratories (Bar Harbor, ME) were kept on a 10-hr dark/14 hr light cycle. Females were bred by placing them with males at the end of the light cycle from 8:00–10:00. The day seminal plugs were found was designated GD 0. Dams were assigned to dosage groups on GD 6 such that dam weights were similar among groups. Extra animals were assigned to the control group to get a good measure of the known background incidence of eye defects in C57BL/6J mice (see http://jaxmice.jax.org/library/notes/463a.html). On GD 7, gravid females were given a total dosage of 0, 3.4, or 4.9 g/kg methanol in distilled water, split into two doses administered by i.p. injection at 9:00 and 13:00. This dosing regimen was based on previous studies of ethanol teratogenesis by Sulik et al. (see above). Actual dosages of methanol were chosen based on preliminary dosage range-finding studies (data not shown). Dams were weighed at several time points after dosing and were killed on GD 7, 8, or 9 for examination of embryos and in situ hybridization (see below) or on GD 17 for litter evaluations. On GD 17, dams were weighed, gravid uteri were removed and weighed, and the numbers of live, dead, and resorbed conceptuses were recorded. Fetuses were removed, weighed as a litter, examined externally, and placed in 95% ethanol. Fetuses were then skinned and eviscerated, stained with Alcian blue for cartilage and Alizarin red for bone and macerated with 2% NaOH. Stained skeletons were stored in 7:3 95% ethanol:glycerin. All fetuses were examined for skeletal morphology using an Olympus SZH stereo dissecting microscope.

Examination of Embryo Morphology on GD 7–9

On the appropriate day of gestation, some dams in the control and high methanol dosage groups were killed by cervical dislocation, the uterus was exposed through a midline incision, and embryos were dissected free from maternal decidual and extra embryonic membranes. Embryos were immediately examined with an Olympus SZH stereo dissecting microscope. Some embryos were photographed with an attached Olympus OM-2S 35 mm camera. Other embryos were fixed in Bodian's solution for at least 24 hr and stained with Acridine orange (Molecular Probes, Eugene, OR) for topographical analysis by confocal laser scanning microscopy using the method of Zucker et al. (1995) and Zucker and Rogers (2000). With this approach, the specimen is rendered opaque by heavy surface staining, and viewing with a fluorescence microscope or confocal laser scanning microscope gives an image similar to a scanning electron micrograph. Briefly, a stock solution of 1 mg/ml of Acridine orange in distilled water is diluted to 0.1 μg/ml with PBS. Fixed embryos were stained in this solution for 5 min and then washed twice in PBS to remove excess stain. After staining, embryos were observed by suspending them in a hanging-drop depression slide and sealing the top with a cover slip. Embryos were imaged using a Leica TCS4D confocal laser scanning microscope (Leica Microsystems, Deerfield, IL) with an Omnichrome laser exciting at 488-nm and emitted light was obtained through a 522/22 filter (Chroma, Brattleboro, VT). Embryos were optically sectioned at 20 μm and the stack of confocal images was combined using a Leica 3-D program.

In Situ Hybridization

To aid in the analysis of head morphology, we used the expression of *en-1* and *hoxb-1* to mark specific cephalic neural regions in the embryo on GD 8. At this stage of mouse development, *hoxb-1* is expressed in presumptive rhombomere 4 (Murphy et al., 1989; Frohman et al., 1990; Murphy and Hill, 1991; Conlon and Rossant, 1992), and *en-1* is expressed in the presumptive mesencephalon and anterior metencephalon (Davis and Joyner, 1988; Davison et al., 1988; McMahon et al., 1992; Joyner, 1996). The probe for *hoxb-1* was provided by M. Frohman (Frohman et al., 1993) and the probe for *en-1* was provided by A. Joyner (Hanks et al., 1995). Whole-mount in situ hybridizations were conducted with digoxigenin labeled antisense riboprobe as described by Conlon and Rossant (1992).

Statistics

All statistical analyses were carried out using procedures available in the SAS system (SAS, 1990). The pregnant dam and the litter were considered the statistical units for all comparisons. Continuous variables were analyzed using the General Linear Models procedure (PROC GLM) and multiple *t*-test of least squares means (LSMEANS) for contrasts of dose group means with controls. Dichotomous variables were analyzed using Fisher's exact test.

RESULTS

Maternal and Litter Parameters

There were no maternal deaths due to methanol exposure in this study. The higher dosage level of MeOH caused maternal weight loss during the 24 hr after dosing (Table 1). By 48 hr after dosing, and for the remainder of pregnancy, weight gain was similar in all groups. The dosages of methanol administered here caused no apparent sedation or other clinical signs of intoxication or toxicity in pregnant mice. The numbers of live fetuses were lower, and the numbers of resorptions higher per litter at both dosages of methanol compared to controls. About half of each litter was resorbed at the high dosage level. No whole-litter losses were observed. Fetus weight was affected only at the high dosage level of MeOH.

Fetal Examinations

Facial dysmorphogenesis. Results of the external craniofacial examinations are presented in Table 2. At the high MeOH dosage level, 84.2% of fetuses per litter exhibited anophthalmia or microphthalmia, and 91.0% of fetuses per litter exhibited at least one craniofacial malformation. Examples of the malformations observed are presented in Figure 1 and include severe holoprosencephaly, facial clefts, and frank facial agenesis. At the lower MeOH dosage, 44% of the fetuses per litter were affected by micro- or anophthalmia and 55.8% per litter exhibited craniofacial defects. There is a significant background incidence of eye defects including microphthalmia in the C57BL/6J mouse strain (see http://jaxmice.jax.org/library/notes/463a.html), with reported values ranging from 4.4% (Chase, 1942) to 10% (Kalter, 1968).

Skeletal defects. Methanol-related effects on skeletal development were limited to the craniofacial skeleton, the cervical vertebrae, and supernumerary lumbar ribs (Table 3). Consistent with the severe craniofacial malformations observed externally, malformations of the facial bones were observed in 51.2% of the fetuses at the high dosage of methanol, and exoccipital fusions were observed in 73.7% of the fetuses in these litters. Cervical vertebral malformations observed in the high methanol dosage group included fusions, splits and duplication of the atlas, axis and C3, tubercula anterior displaced from C6 to C5, and rib on C7. Supernumerary lumbar ribs and <26 presacral vertebrae were also observed in this group. In the lower methanol dosage group, significant increases were noted in split axis, fused C3, missing vertebrae in C3–C5, supernumerary lumbar rib, and <26 presacral vertebrae (Table 3).

Embryo Morphology

External morphology. At the late headfold stage, approximately 16 hr after the second maternal dose of methanol, the anterior neural plate of methanol-treated embryos appeared narrower in the area of the future prosencephalon than did controls (Fig. 2A,B). At the 6–8 somite stage (24–28 hr after dosing), the anterior neural plate remained narrow compared to controls, especially in the prosencephalon (Fig. 2C,D). By GD 9 (18–22

Table 1
Maternal and Litter Parameters[a]

	Methanol (g/kg)		
Parameter	0	3.4	4.9
Pregnant at term (*n*)	43	13	24
Weight gain GD 7–8 (g)	0.33±0.10	0.37±0.15	−0.24±0.14[b]
Weight gain GD 7–10 (g)	1.63±0.18	2.20±0.20	1.50±0.20
Live fetuses/litter (*n*)	7.5±0.3	6.3±0.5[b]	3.7±0.4[b]
Resorbed/litter (*n*)	0.4±0.1	1.3±0.4[b]	4.4±0.4[b]
Dead/litter (*n*)	0.1±0.1	0±0	0.1±0.1
Fetus weight (g)	0.83±0.02	0.82±0.03	0.70±0.02[b]

[a]Data are means±SEM. Maternal weight gain was affected 24 hr after dosing only at the high dosage level of methanol. Significant embryo/fetal mortality was observed at both dosage levels and fetus weight at term was reduced at the high dosage.
[b]Significantly different from control value, $p<0.05$.

Table 2
External Craniofacial Findings[a]

	Methanol (g/kg)		
Finding	0	3.4	4.9
Litters/fetuses (*n*)	43/343	13/75	24/90
Micro/anophthalmia	8/12 (3.8)	9/34 (44.0)[b]	21/72 (84.2)[b]
Low-set/malformed ears	1/1 (0.3)	3/9 (10.9)	21/59 (73.7)[b]
Single/missing nares	0/0 (0)	4/4 (5.8)	18/42 (47.7)[b]
Maxilla malformed	1/1 (0.5)	7/13 (16.9)[b]	19/60 (61.6)[b]
Mandible malformed	1/1 (0.3)	3/3 (3.1)	20/54 (62.1)[b]
Severe facial dysgenesis[c]	0/0 (0)	1/1 (0.9)	15/25 (31.2)[b]
Facial defect (any)[d]	10/14 (4.4)	11/44 (55.8)[b]	23/77 (91.0)[b]

[a]Both dosages of methanol produced significant increases in craniofacial malformations with the higher dosage producing such malformations in a mean of 91% of fetuses per litter. Values represent the number of litters/numbers of fetuses affected, followed by the mean % per litter affected.
[b]Significantly different from control value, $p < 0.05$.
[c]See Figure 1D,F,H.
[d]Includes micro/anophthalmia.

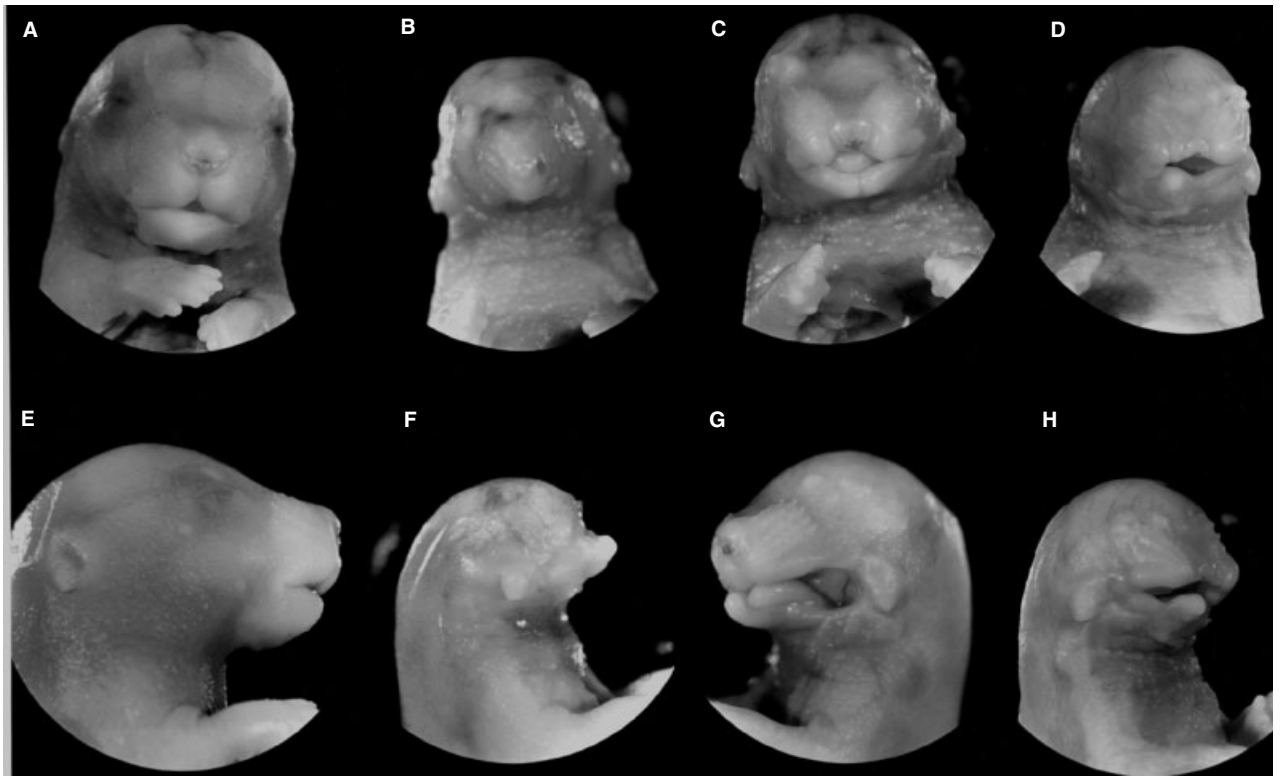

Fig. 1. Representative range of facial dysmorphologies in GD 17 fetuses of dams dosed with MeOH on GD 7. Controls are shown in front (top row) and side (bottom row) views. Dysmorphologies observed include holoprosencephaly with single naris and micrognathia (B), cleft lip (**C**), varying degrees of maxillary and mandibular hypoplasia (B– **D**, **F**, **H**), lateral facial cleft (**G**), and low-set ears (B–D, F–H). Gross facial dysgenesis was apparent in some fetuses (D,F,H).

somites), the forebrains of methanol-treated embryos were clearly dysmorphic, with small or missing optic vesicles and small telencephalons. Treated embryos also exhibited dysmorphology of the branchial arches which were hypoplastic and poorly separated compared to controls (Fig. 2E,F). Holoprosencephaly with severe forebrain hypoplasia was clearly evident in some treated embryos by GD 9 (Fig. 3).

Expression Domains of *Hoxb-1* and *En-1*

Hoxb-1 and *en-1* were expressed in rhombomere four and in the mesencephalon, respectively, in both control and methanol-treated embryos. The expression domains for both *hoxb-1* and *en-1* mark these brain structures and demonstrate the shortening of the forebrain in methanol-treated embryos (Fig. 4). Although the expression

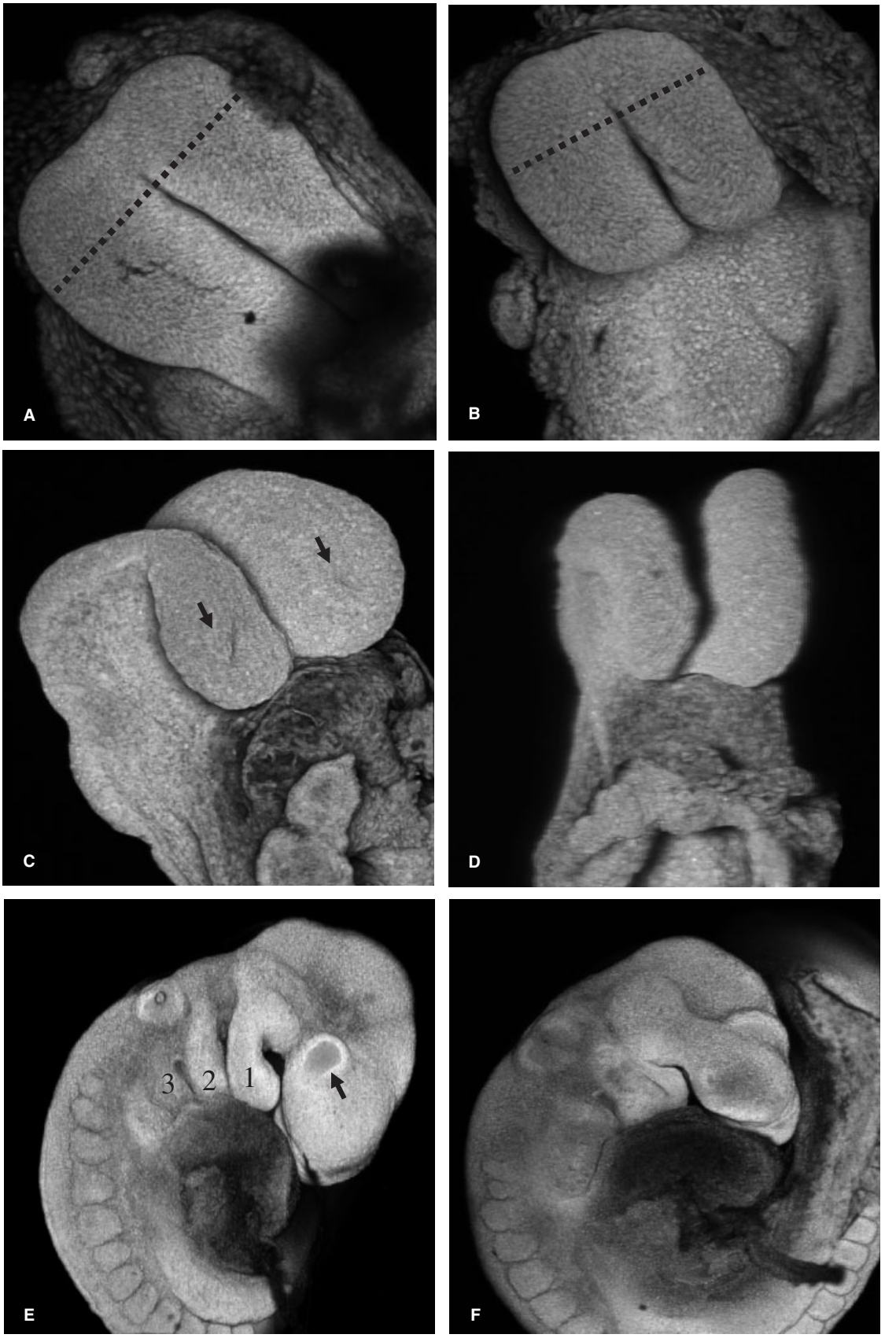

Fig. 2.

Table 3
Skeletal Findings[a]

Finding	Methanol (g/kg)		
	0	3.4	4.9
Litters/fetuses examined	40/292	12/68	23/81
Missing/malformed facial bones	0/0 (0)	1/1 (1.0)	16/40 (51.2)[b]
Exoccipital fusion[c]	0/0 (0)	2/2 (3.1)	11/16 (73.7)[b]
Atlas split	1/1 (0.3)	1/1 (1.4)	7/10 (8.7)[b]
Atlas fused[c]	0/0 (0)	5/6 (10.0)	16/36 (43.9)[b]
Atlas duplicated	0/0 (0)	0/0 (0)	5/5 (12.3)[b]
Axis split	2/2 (0.7)	6/18 (25.7)[b]	14/25 (27.8)[b]
Axis fused[c]	1/1 (0.3)	3/6 (11.4)	12/32 (35.7)[b]
C3 Split	0/0 (0)	0/0 (0)	4/6 (6.2)[b]
C3 Fused	1/1 (0.3)	2/5 (9.7)[b]	5/9 (9.5)[b]
Missing C3–C5 (any)	2/2 (0.8)	9/35 (51.8)[b]	14/33 (47.5)[b]
Tuberculum anterior on C5	0/0 (0)	2/2 (2.1)	2/4 (5.4)[b]
Rib on C7[d]	4/4 (1.4)	2/2 (2.3)	6/13 (18.1)[b]
Rib on L1[d]	0/0 (0)	8/11 (10.1)[b]	6/10 (16.2)[b]
<26 presacral vertebrae	6/6 (2.6)	11/42 (61.4)[b]	15/35 (47.9)[b]
Any skeletal anomaly	12/14 (5.3)	12/61 (91.1)[b]	20/66 (86.4)[b]

[a]Both dosages of methanol produced significant increases in skeletal malformations, principally in the cranial and cervical regions. Values represent the number of litters/number of fetuses affected, followed by the mean % per litter affected. There was a wide morphological variation in the types and extent of splits and fusions observed.
[b]Significantly different from control value, $p < 0.05$.
[c]Fused with adjacent bones (i.e., exoccipital/atlas, atlas/axis).
[d]Does not include pinpoint ossifications.

domain for *hoxb-1* seemed to be in rhombomere four in both methanol-treated and control embryos, the length of the neural tube anterior to this expression domain was shortened in methanol treated embryos (Fig. 4A,B). More strikingly, the expression of *en-1* demarcates the mesencephalon and demonstrates the gross prosencephalic hypoplasia in methanol-treated embryos (Fig. 4C,D).

DISCUSSION

Treatment of pregnant C57BL/6J mice with methanol on GD7 produced craniofacial malformations similar to the FAS-like facies produced by ethanol. Affected fetuses exhibited anophthalmia, microphthalmia, varying degrees of holoprosencephaly, and associated ear and jaw malformations. These results suggest that these two alcohols have common targets and may share common modes of action in their teratogenicity. Although the dosages of methanol used (3.4 and 4.9 g/kg) are high, they are lower than those (two doses totaling 5.8 g/kg) typically used in ethanol pathogenesis studies in C57BL/6J mice (e.g., Dunty and Sulik, 2002; Sulik et al., 1981, 1984, 1988; Sulik and Johnson 1983; Webster et al., 1983, 1984; Webster and Ritchie, 1991; Kotch and Sulik, 1995). The lower dosage of methanol required to produce craniofacial malformations, and the almost complete facial agenesis observed in some of the fetuses from methanol-treated dams, suggest that methanol is a relatively more potent teratogen than is ethanol when administered during gastrulation. There are few published studies of ethanol teratogenesis in CD-1 mice, so the specific malformations that might be induced by ethanol in this strain have not been determined.

Observations of embryos in the hours after methanol administration indicate that the cell movements and proliferation characteristic of gastrulation may be impeded by methanol, leading to the anterior deficiencies seen in these embryos and fetuses. By GD 9, anophthalmia and holoprosencephaly are clearly apparent in some methanol-treated embryos, as are malformations of the branchial arches. Similar pathogenesis has been postulated for ethanol (Nakatsuji and Johnson, 1984; Sulik, 1984; Sanders et al., 1987). Both ethanol (Dunty and Sulik, 2002) and methanol (Degitz et al., 2003) cause malformations of the branchial arches and associated cranial ganglia and nerves.

Treatment of pregnant CD-1 mice with methanol by inhalation or oral gavage during gestation results in exencephaly, cleft palate, and cervical skeletal malformations (Rogers et al., 1993; Rogers and Mole, 1997; Connelly and Rogers, 1997). Neither cleft palate nor exencephaly were observed in the present studies in C57BL/6J mice. The types of craniofacial malformations produced by methanol in these two strains of mice are

Fig. 2. Control embryos (**A**, **C**, **E**) and embryos of dams treated with 4.9 g/kg methanol, split in two doses on GD 7 (**B**, **D**, **F**). Embryos are shown at GD 7, approximately 6 hr after the second dose (A,B), GD 8 (C,D), and GD 9 (E,F). Compared to controls, methanol-treated embryos exhibited narrower anterior neural plate on GD 7 (B vs. A, dotted lines), narrowed prosencephalic folds with missing optic pits on GD 8 (D vs. C, optic pits at arrows) and apparent anophthalmia, prosencephalic deficiency and dysmorphic branchial arches on GD 9 (F vs. E. Eye at arrow, branchial arches 1–3 indicated in E). Embryos were fixed overnight in Bodian's solution, surface stained with acridine orange, and imaged by confocal microscopy.

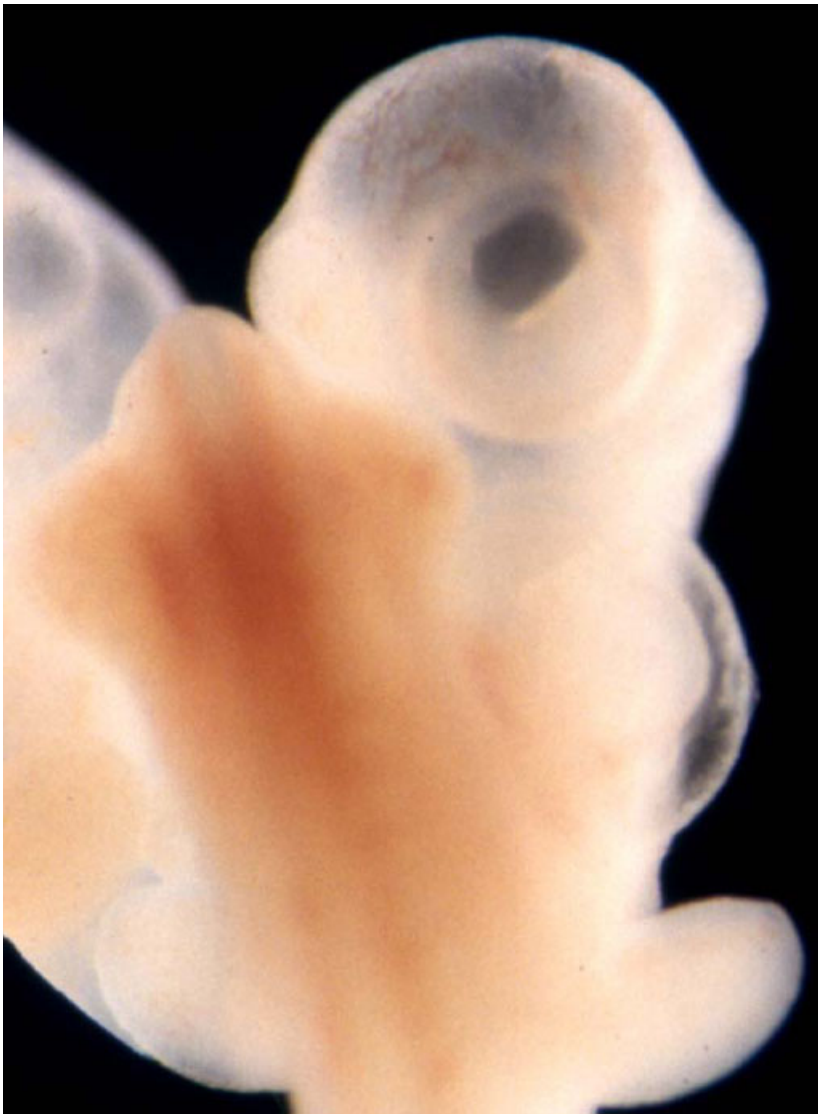

Fig. 3. Frontal view of GD 9 embryo from a dam treated with 4.9 g/kg methanol on GD 7. A single small prosencephalon (holoprosencephaly) is clearly evident (arrow).

different. Although the route of administration (i.p. in C57BL/6J vs. inhalation or oral in CD-1) was different, methanol would be expected to rapidly distribute with either route of administration. Whereas differences in pharmacokinetics cannot be ruled out, these findings suggest different vulnerabilities of the two strains to methanol during craniofacial morphogenesis. In contrast, the cervical skeleton malformations observed in C57BL/6J fetuses in the present study are the same as those observed in the CD-1 methanol studies cited above. These consist of malformations consistent with homeotic posteriorization of the cervical vertebrae (Connelly and Rogers, 1997), including ribs on C7 and tubercula anterior on C5. The cervical skeleton malformations produced by methanol in both of these strains of mice are possibly due to deficiencies of paraxial mesoderm, the cells of which are just beginning to emerge at the point in gastrulation at which exposure occurred.

The results presented demonstrate that methanol produces severe craniofacial malformations in C57BL/6J mice. These malformations are similar to those produced by ethanol, suggesting that these two alcohols affect the same cell populations. That methanol and ethanol are closely related alcohols and produce very similar effects further suggests that they may work though similar modes of action. The extant literature on ethanol includes evidence for a variety of toxic mechanisms, including but not limited to apoptosis, membrane effects, oxidative damage, and interference with retinoic acid metabolism. Whereas there are far fewer studies on methanol teratogenesis, similar toxic mechanisms may be involved (for review see Rogers and Daston, 1997). Ethanol is a known human teratogen at high dosages. Although the findings presented here suggest a similar potential for methanol, exposures to methanol in the range of those used here are highly unlikely in humans.

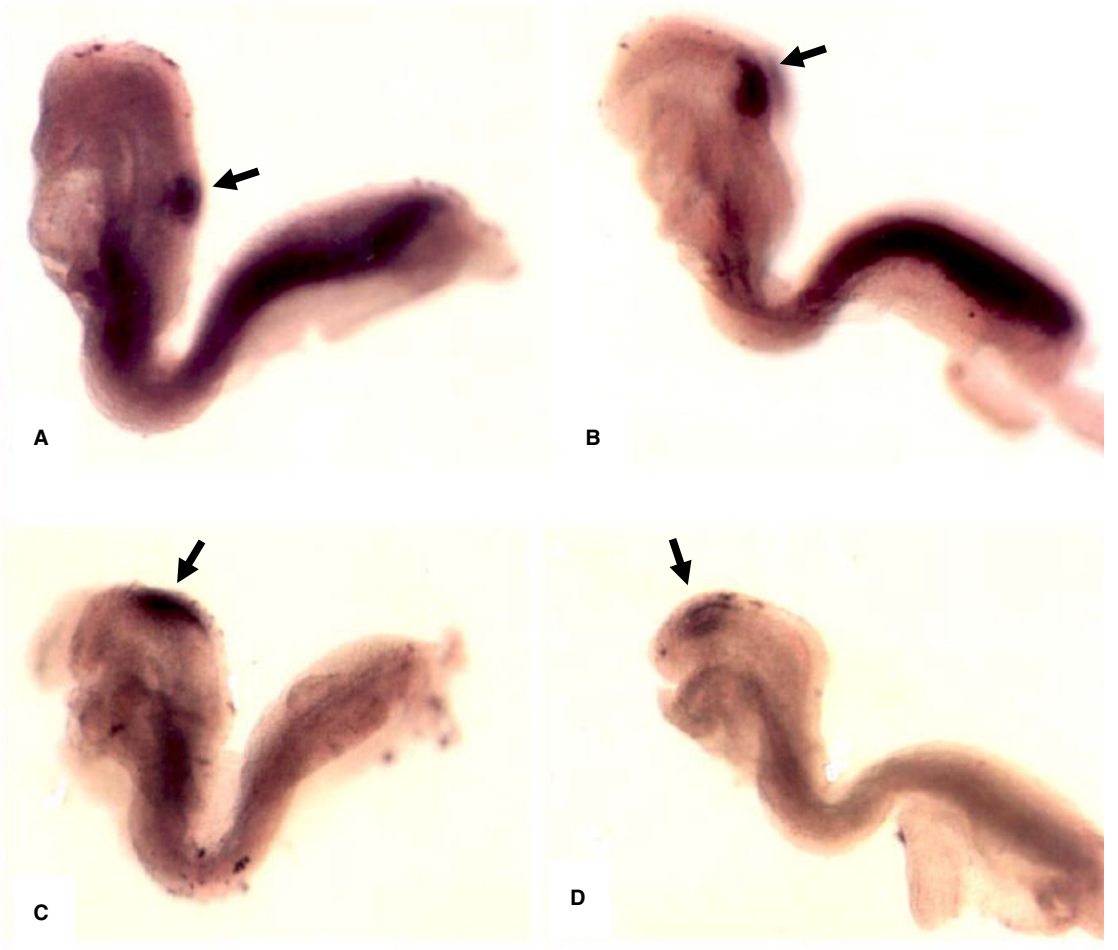

Fig. 4. In situ hybridizations for *hoxb-1* (**A**, **B**) and *en-1* (**C**, **D**) gene expression in GD 8 control embryos (A,C) and embryos from a dam treated with 4.9 g/kg methanol on GD 7. *Hoxb-1* is expressed in rhombomere 4 of the hindbrain (arrows in A,B) and *en-1* is expressed in the midbrain and anterior hindbrain (arrows in C,D). These expression domains are morphological landmarks demonstrating the deficiency of brain structures anterior to them in embryos of methanol-treated dams compared to controls.

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