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INFLUENCE OF DIETARY FOLIC ACID ON THE DEVELOPMENTAL TOXICITY OF METHANOL AND THE FREQUENCY OF CHROMOSOMAL BREAKAGE IN THE CD-1 MOUSE

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Abstract — The proposed increased use of methanol (MeOH)-based fuels raises the concern for an increased risk for MeOH toxicity. MeOH, which is detoxified in part via a folate-dependent pathway, is known to be teratogenic in rodents. Previous observations have implicated maternal folate status as a critical modulator for the developmental toxicity of MeOH. The current study extends these findings, examining the effect of maternal dietary folate intake on fetal folate stores, as well as identifying a possible marker for the prediction of the developmental toxicity of MeOH. Virgin female CD-1 mice were assigned to diets containing either 400 (marginal) or 1200 (control) nmol folic acid (FA)/kg, and 1% succinylsulfathiazole for 5 weeks prior to mating and throughout breeding and gestation. From gestation day (GD) 6 through 10 dams were given by gavage deionized, distilled water (dH_2O) or MeOH at 2.5 g/kg body weight, twice daily. On GD 18, mice were weighed and killed and the liver, kidneys, and gravid uteri removed and weighed. Implantation sites, live and dead fetuses, and resorptions were counted; fetuses were weighed individually and examined for cleft palate and exencephaly. The marginal FA dietary treatment resulted in low maternal liver (50% reduction) and red cell folate (30% reduction) concentrations, as well as low fetal tissue folate concentrations (60 to 70% reduction) relative to the adequate FA dietary groups. Marginal FA treatment alone resulted in cleft palate in 13% of the litters; there were no litters affected with cleft palate in the adequate FA-control group. Marginal FA-MeOH treatment resulted in a further increase in the litters affected by cleft palate (72% of litters affected). The percent of litters affected by exencephaly was highest in the marginal FA-MeOH group. The frequency of micronuclei in maternal and fetal reticulocytes, a marker for chromosomal abnormalities, was not influenced by either the marginal FA diet or by MeOH treatment. These results show that marginal folate deficiency in pregnant dams significantly increases the teratogenicity of MeOH. © 1996 Elsevier Science Inc.

Key Words: methanol; folic acid; development; nutrition; nutrient–drug interaction; chromosomal breakage; pregnancy; diet.

INTRODUCTION

During the past decade there has been increasing interest in the possible use of methanol (MeOH) as an alternative automotive fuel source. While there are advantages in adapting MeOH-based fuels with respect to air pollution, concern has been expressed over the possibility that the number of cases of MeOH toxicity could rise. Typical signs of acute MeOH toxicity in humans include metabolic acidosis, ocular pathology, and in severe or untreated cases, lethality. MeOH is rapidly absorbed through inhalation, ingestion, or percutaneous exposure (1). In liver, MeOH is rapidly metabolized to formaldehyde and then to formic acid and CO_2 via a catalase–peroxidase enzyme system in rodents, or alcohol dehydrogenase in nonhuman primates and humans (1). The accumulation of formic acid and its dissociation product, formate, in blood and soft tissues following high-dose MeOH administration can contribute to the development of metabolic acidosis. Formate has also been implicated as the agent responsible for MeOH-induced visual damage seen in nonhuman primates (2–6). Unlike humans and other primates, rodents metabolize formate rapidly after MeOH ingestion; therefore, formate does not accumulate and signs of MeOH toxicity are typically not manifested (7). As a consequence of the above it has been argued that rodents are a poor model for humans when it comes to the study of MeOH toxicity. However, if the rate of formic acid oxidation is slowed in rodents; formate concentrations can become elevated and initiate similar pathologies to those observed in primates. While the above inhibition is typically accomplished through

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the use of nitrous oxide (3,8,9), it can also be achieved by inducing a condition of folate deficiency in the animal (9,10) because formate oxidation is dependent on tetrahydrofolate (THF) (9,11).

While the toxic consequences of acute MeOH exposure in humans have long been known (1,12), the potential developmental toxicity of MeOH has only recently been recognized. In 1985, Nelson *et al.* (13) reported that in Sprague–Dawley rats, inhalation of MeOH at high concentrations (10,000 to 20,000 ppm) for 7 h/d throughout gestation induced a dose-related increase in fetal abnormalities. Infurna and Weiss (14) subsequently reported that prenatal MeOH exposure in Long–Evans rats produced behavioral abnormalities in their offspring even when signs of maternal MeOH toxicity were not evident. Rogers et al. (15) reported an increased incidence of fetal abnormalities, including exencephaly, cleft palate, and skeletal malformation, in offspring of CD-1 mice exposed to MeOH by inhalation at doses of 5,000 ppm and above, or by oral administration of 4 g MeOH/kg/d on gestation days (GD) 6 through 15. MeOH also has been shown to be embryotoxic in rodent whole embryo culture with mouse embryos being more sensitive than rat embryos (16–18).

While the above reports clearly establish the potential teratogenicity of MeOH, it is important to note that the levels of exposure needed to influence development are markedly higher than those that would typically occur in the environment. One interpretation of the above observation is that MeOH does not represent a significant reproductive risk for humans. However, prior to the acceptance of this idea, it is important to investigate the possibility that MeOH could interact synergistically with other putative or known teratogens. In this regard, our group has been interested in the hypothesis that the developmental toxicity of MeOH would be amplified under conditions of folate deficiency. Consistent with this idea, we have shown that the teratogenicity of MeOH given orally on GD 6 through 15 (5 g/kg/d) is increased in CD-1 mice when the dams are fed folate deficient diets (19).

The current study was performed to confirm and extend our previous findings on the influence of maternal folate deficiency on MeOH teratogenicity. A key question asked in this work was whether MeOH exposure influenced maternal and/or fetal folate concentrations. A second question addressed was whether the measurement of maternal reticulocyte micronuclei (MN) frequency would be of value in the identification of dams at high risk for MeOH and/or folic acid deficiency-associated teratogenecity. MN arise in cells when whole chromosomes or chromosomal fragments lag at anaphase and fail to incorporate into the daughter cells (20). Frequencies of MN in humans have been reported to be increased under conditions of folate deficiency (21,22), and in mice the cytogenetic damage of folate deficiency has been reported to be enhanced by caffeine (23).

MATERIALS AND METHODS

Materials

Lyophilized cultures of *L. casei* subspecies rhamnosis (ATCC 7469) were obtained from the American Type Culture Collection, Rockville, MD. Lactobacilli MRS broth and folic acid casei medium were purchased from Difco Laboratories, Detroit, MI. L-Sodium ascorbate, HEPES, CHES, acridine orange, and folic acid were from Sigma Chemical Co., St. Louis, MO. Methanol (Optima HPLC Grade), β-mercaptoethanol, glycerol, sodium acetate, sodium phosphate dibasic, and monobasic were purchased from Fisher Scientific Co., Pittsburgh, PA. Microtiter plates and 0.22 μm filters were obtained from Corning and Costar Corp., New York, NY.

ANIMALS

Weanling CD-1 female mice (Charles Rivers, Inc., Gilroy, CA) weighing 12 to 14 g were housed in stainless steel, wire-bottomed cages in a room with a 12-h light/dark cycle at 20 to 23°C and 50% humidity. Animals were fed an amino acid-based, folic acid-free diet supplemented with either 400 or 1200 nmol folic acid/kg diet and 1% succinylsulfathiazole for 5 weeks prior to mating and throughout breeding and gestation (24).

Breeding. Males from the same strain were placed with females (two or three per cage) in breeding cages at 2000 h and removed the next morning at 0800 h. The presence of a vaginal plug was considered to be evidence of mating. The morning of the day that the plug was found was denoted gestation day 0 (GD 0). Pregnant mice were ear-marked and placed in new cages. No more than three pregnant mice were placed in the same cage. If no plug was present, the animal was bred again; the breeding period was continued for up to 10 d. Animals not bred by day 10 were removed from the study. Pregnant animals were weighed on GD 0, 5, 10, 12, 15, and 18.

Methanol Treatment. The pregnant animals were exposed to MeOH by gavage at a dosage of 2.5 g/kg (15.65% Optima HPLC grade MeOH in deionized water (dH_2O)), given twice daily, from GD 6 to GD 10; control animals were given equivalent volumes of dH_2O by gavage. The dose of MeOH was based on previous work in our laboratory indicating that this dose and regime resulted in developmental abnormalities in the CD-1 mouse (19).

Blood and Tissue Collection. On GD 18, animals were anesthetized with CO_2, and exsanguinated by cardiac puncture. Cardiac blood was collected using heparinized syringes. An aliquot of whole blood (~ 50 μL) was drawn into a microhematocrit tube for hematocrit determination. A second aliquot (100 μL) of whole blood was immediately transferred into an eppendorf tube and stored at −20°C until analyzed for red cell folate concentration. The remaining blood was centrifuged and plasma was transferred to tubes and stored at −80°C until analyzed for folate.

Maternal liver and kidneys were rapidly excised, weighed, frozen in liquid nitrogen, and stored at −80°C until assayed for folate concentrations. The uterus was excised and weighed, and the number and position of fetuses (live and dead) and resorption sites were recorded. Live fetuses were weighed, measured for crown–rump lengths, and examined for the presence of external malformations. Fetal livers were then collected, frozen with liquid nitrogen, and stored at −80°C until analyzed for folate concentration.

Micronucleus Assay. Maternal tail vein blood (2 to 3 μL) was collected on precleaned microscope slides 48 h after the final MeOH treatment. On GD 18 blood smears were also made from 2 to 3 μL of fetal blood on precleaned microscope slides. Slides were air dried and fixed in absolute methanol on the same day. Fixed slides were stained with 0.1% acridine orange for 5 min, followed by a 17-min rinse in Sorensen's M/15 phosphate buffer, pH 6.8 (25). Slides were then wet mounted with cover slips (1-1/2 mm thick) and examined by fluorescent microscopy using a mercury lamp. Under these conditions, reticulocytes (containing RNA) fluoresce red while micronuclei (containing DNA) fluoresce green-yellow. The frequency of micronuclei was manually scored in 1,000 reticulocytes per slide using an Olympus IMT-2 microscope at 1,000 × under oil immersion.

Tissue analysis

Extraction of Folates from Maternal and Fetal Livers. Extraction of tissue folates was based on the method of Horne *et al.* (26). Briefly, tissues were boiled in 10 vol of extraction buffer for 10 min in the dark. The extraction buffer, which consisted of 2% (w/v) sodium ascorbate, 0.2 M β-mercaptoethanol, 50 mM HEPES, and 50 mM CHES, was boiled prior to use. Cooled liver tissues were homogenized with a Teflon-glass tight fitting tissue homogenizer. Liver homogenates were centrifuged at 40,000 × *g* for 20 min at 4°C. The supernate was removed and centrifuged again for another 10 min at 40,000 × *g* at 4°C. The lipid layer was removed carefully by aspiration. Liver extracts were filtered through a 0.22 μm membrane and stored under an atmosphere of argon at −80°C.

Extraction of Folate from Whole Blood and Plasma. Red blood cell and plasma folate extractions were prepared as described by Heid *et al.* (27). Frozen blood was thawed and diluted with 1.4 mL of 0.05 mM phosphate buffer (pH 6.1) and 0.2% ascorbate. Diluted blood samples were autoclaved for 10 min at 121°C to deproteinize the samples; the blood was then cooled and centrifuged at 10,000 × *g* for 15 min. Clear supernates were stored at −20°C.

Frozen plasma was thawed, and a 100 μL aliquot was diluted with 400 μL of ascorbate containing phosphate buffer, mixed, and autoclaved at 121°C for 10 min to deproteinize the plasma. Samples were cooled and centrifuged at 10,000 × *g* for 15 min. Clear supernates were collected and stored at −20°C.

Treatment of Liver Extracts With Rat Serum Conjugase. One drop of toluene and 0.25 vol of rat serum conjugase were added to filtered liver extracts. Samples were incubated at 37°C for 3 h under an atmosphere of argon to convert polyglutamates to di- or monoglutamates (28).

Treatment of Red Cell Folate With Hog Kidney Conjugase. Frozen supernatant extracts of whole blood and plasma were thawed and 250 μL aliquots were transferred to tubes containing 2.25 mL 1% ascorbate (pH 4.8), 2.25 mL 0.1 M sodium acetate buffer (pH 4.8), and 250 μL of folate-free hog kidney conjugase (29). Samples were mixed and the mixture was incubated at 37°C for 6 h to convert polyglutamates to di- or monoglutamates. Following incubation, samples were stored at −20°C until analyzed for folate.

Preparation of Rat Serum Conjugase. Blood was drawn by cardiac puncture from male Sprague–Dawley rats after exposure to CO_2. Blood was allowed to clot at room temperature for 20 min. The clot was carefully removed and the serum was centrifuged at 5,000 × *g* at 4°C for 10 min. The dialysis membrane (MWCO 12,000–14,000) was boiled in 1 mM EDTA solution for 10 min. The membrane was cooled and rinsed thoroughly both inside and outside of the tubing with deionized water. About 10 mL of serum was dialyzed at 4°C for 24 h against 1 L of 0.1 M potassium phosphate buffer, pH 7.0 containing 2 g/L activated charcoal, to remove endogenous folates. The dialysis buffer was changed after 24 h without addition of charcoal and dialyzed for another 48 h with several changes. The dialyzed serum was stored at −20°C for later use. The concentration of folate in the conjugase, as measured by *L. casei* assay, was less than 10 ng/mL total folate.

Preparation of Hog Kidney Conjugase. Fresh hog kidney (~ 200 g) with fat removed was homogenized in 400 mL of 0.32% cysteine HCl solution and made up to a final volume of 700 mL. This homogenate was autolyzed for 2 h at 37°C. After the incubation, the homogenate was centrifuged for about 20 min at 1,000 × *g* at 4°C. The supernate was centrifuged for another 30 min at 4,000 × *g* at 4°C. The pH of the supernate was adjusted to 4.5 with 1 N HCl. This solution was treated with 30 g of Dowex (1 × 8 Chloride) in an ice bath for 1 h to remove any endogenous folate associated with the hog kidney conjugase. After the treatment, the solution was filtered through glass wool. The clear filtrate was stored in 5 mL aliquots at −20°C until further purification of enzyme. After thawing, the aliquot was applied to a Sephadex G-25 column (1.5 × 30 cm) with the elution buffer composed of 0.1 M acetate buffer, pH 4.8, containing 0.32% cysteine HCl. Purified enzyme was collected in 10 mL after eluting 17 mL of buffer. Fractions of purified hog kidney conjugase were stored at −20°C for up to 3 months.

Microbiologic folate assay

Glycerol-Cryoprotected L. casei. An *L. casei* inoculum was prepared by a modification of the method of Wilson and Horne (28). Lyophilized *L. casei* was suspended in 1 mL of Lactobacilli MRS broth. Half of the bacterial suspension was inoculated in 10 mL Lactobacilli MRS broth at 37°C for at least 18 h. A small aliquot of this inoculum (0.5 mL) was added to a folic acid casei medium that was prepared by dissolving 9.4 g of powdered medium and 50 mg sodium ascorbate in 200 mL of dH_2O. To this 0.3 μg/L of folic acid was added. The solution was autoclaved in the flask at 121°C for 10 min. Bacteria were inoculated in folic acid casei medium for 18 h at 37°C, after which the culture was cooled in an ice water bath. An equivalent volume of cold, sterile 80% glycerol was added to the chilled culture; the resulting mixture was stored in 2 mL aliquots in sterile cryovials at −80°C.

Folic Acid Standard. A folic acid standard was prepared by dissolving 8.8 mg of folic acid in 100 mL of 20% ethanol solution containing 1 mL 0.1 N NaOH. Folic acid standard was diluted to 100 mL with dH_2O and stored in 1-mL aliquots in eppendorf tubes at −20°C. The resulting folic acid concentration in the vials was 200 nmol/mL. On the day of assay, folic acid standard was diluted with dH_2O to a final concentration of 2 pmol/mL. The standards were sterilized by filtration.

Assay Procedures. Assays were performed in 96-well microtiter plates following the procedure developed by Horne and Patterson (30). Folic acid casei medium was prepared as described in the package insert and sterilized by filtering through a 0.22 μm membrane into a sterile container.

The *L. casei* inoculum was prepared by mixing 1 vol of the thawed bacterial suspension with 19 vol of sterile 0.9% sodium chloride solution.

The working buffer was prepared as follows: 3.2 g of sodium ascorbate were dissolved in 19 mL of dH_2O; 1 mL of 1 M potassium phosphate buffer (pH 6.1) was added and the solution was filtered into a sterile container using a 0.22 μm sterile syringe filter.

Each well of the microtiter plate contained 8 μL of the working buffer. The standard curve ranged from 20 to 120 fmol of folic acid in triplicate, with a maximum volume of 60 μL. The volumes of standards and conjugase-treated samples were adjusted to 150 μL with sterile dH_2O. Folic acid casei medium (150 μL) was added and then 20 μL of the *L. casei.* The plate was incubated for 18 h in an oven at 37°C and then read at 600 nm in a ELISA Plate Reader (SLT-labinstruments, Austria).

Statistical analysis

For all statistical analyses the pregnant dam and the litter were considered the units for comparisons. Continuous variables were analyzed using the two-way analysis of variance procedure and the Fisher PLSD for multiple comparisons of means. These analyses were carried out on STATVIEW SE+ (Abacus, Berkeley CA). Incidences of fetal malformations and frequency of micronuclei, based on affected litters, were analyzed using binomial statistics (31).

RESULTS

Net maternal weight gain was not affected by dietary folic acid or MeOH treatment (Table 1). Maternal body weights were similar among the groups throughout gestation with the exception that on GD 18, dams fed adequate folic acid and treated with water had higher body weights than the marginal folic acid–water group. Nongravid maternal body weights were similar among the groups.

There was a significant interaction ($P < 0.05$) between folate and MeOH with respect to relative maternal liver weight. The marginal folic acid dietary treatment was associated with an increase in relative maternal liver weights, the highest values occurring in dams exposed to MeOH (Table 1). Relative kidney weights and relative gravid uterus weights were not consistently affected by either treatment; however, consistent with previous findings (19), the highest relative kidney weights were observed in the marginal folic acid–MeOH group. There was a significant interaction ($P < 0.05$) between folate and MeOH with respect to relative gravid uterus weights.

Table 1. The influence of dietary folic acid intake (400 and 1200 nmol folic acid/kg diet) on the effects of methanol on maternal parameters

	Marginal folate		Adequate folate	
	Water	MeOH	Water	MeOH
Litters (n)	(22)	(22)	(24)	(21)
Maternal weight (g)				
Day 0	26.59 ± 0.63[a]	26.99 ± 0.57[a]	25.72 ± 0.48[a]	27.19 ± 0.73[a]
Day 5	28.71 ± 0.70[a]	29.33 ± 0.61[a]	28.03 ± 0.54[a]	28.81 ± 0.53[a]
Day 10	31.40 ± 0.76[a]	31.05 ± 0.47[a]	31.13 ± 0.56[a]	31.28 ± 0.55[a]
Day 12	34.48 ± 0.73[a]	34.70 ± 0.53[a]	34.39 ± 0.72[a]	34.82 ± 0.65[a]
Day 15	40.33 ± 0.98[a]	40.41 ± 0.55[a]	41.05 ± 0.98[a]	41.08 ± 0.99[a]
Day 18	45.57 ± 1.00[b]	46.83 ± 0.78[a,b]	49.34 ± 1.16[a]	47.47 ± 1.60[a,b]
Nongravid maternal body weight, GD 18 (g)	31.52 ± 0.92[a]	31.03 ± 0.55[a]	31.15 ± 0.66[a]	31.46 ± 1.09[a]
Net maternal weight gain (g)	4.93 ± 0.85[a]	4.04 ± 0.34[a]	5.44 ± 0.54[a]	4.27 ± 1.27[a]
Relative tissue weights, GD 18 (% nongravid maternal weight)				
Liver	7.54 ± 0.21[b]	8.15 ± 0.22[c]	6.44 ± 0.12[a]	6.31 ± 0.15[a]
Kidney	1.41 ± 0.04[a]	1.53 ± 0.04[b]	1.44 ± 0.04[a,b]	1.44 ± 0.04[a,b]
Gravid uterus	46.03 ± 3.42[b]	51.25 ± 1.89[a,b]	58.49 ± 1.97[a]	51.55 ± 3.14[a,b]
Mean hematocrit (%)	40.76 ± 0.77[a]	40.37 ± 0.93[a]	42.08 ± 0.59[a]	42.21 ± 0.66[a]
Maternal liver folate	4.65 ± 0.37[b]	4.55 ± 0.19[b]	9.54 ± 0.50[a]	9.26 ± 0.42[a]
Total maternal liver folate	12.88 ± 0.85[b]	11.60 ± 0.59[b]	17.72 ± 1.41[a]	17.14 ± 1.43[a]
Maternal plasma folate	14.06 ± 1.59[b,c]	10.85 ± 1.37[b]	20.09 ± 2.75[a]	16.50 ± 1.84[a,c]
Maternal red cell folate	610.15 ± 40.12[b]	633.93 ± 29.89[b]	902.25 ± 55.95[a]	896.63 ± 73.76[a]

Methanol was given orally at a dose of 2.5 g/kg body weight, twice daily, between gestation days 6 and 10. Number in parentheses is the number of dams. Values are expressed as mean ± SEM, using litter as the unit of comparison. Values in a row with different superscript are significantly different ($P < 0.05$).

Low dietary folic acid was associated with low relative gravid uterus weight in the water treated groups; however, this effect was not evident in the MeOH-treated groups. Further, there was no effect of MeOH on the relative gravid uterus weight.

Maternal plasma folate concentrations in the marginal folic acid groups were lower than in their respective controls (Table 1); the lowest values were in the marginal folic acid–MeOH group. Maternal erythrocyte and hepatic folate concentrations were significantly lower in the marginal folic acid groups than in their respective controls. The low hepatic folate concentrations observed in the marginal folic acid groups were not due to liver hypertrophy, as total liver folate content was also significantly lower in this group compared to controls. Maternal tissue folate concentrations were not significantly influenced by MeOH treatment; however, there was a trend toward lower plasma folate concentrations ($P = 0.08$) in both dietary groups following MeOH treatment. The changes in plasma, erythrocyte, and hepatic folate concentrations in the marginal dietary folic acid groups were accompanied by a decrease in maternal hematocrit, providing evidence that a functional folate deficiency had been induced.

Both low dietary folate and MeOH exposure negatively affected reproductive outcome (Table 2). While the number of implantation sites was similar among the groups, there was a significant interaction between folate and MeOH with respect to frequency of resorption ($P < 0.01$), fetal death ($P < 0.05$), and fetal malformation ($P < 0.01$). The percent live fetuses/litter was highest in the adequate folic acid–water group. The number of malformed fetuses/litter was highest ($P < 0.05$) in the marginal folic acid–MeOH group. Total sites affected (including resorbed, dead, and malformed fetuses) were significantly affected by both MeOH ($P < 0.05$) and folate ($P < 0.05$) treatments, the highest occurrence being in the marginal folic acid–MeOH group.

MeOH exposure was associated with lower fetal weights in the adequate folic acid group ($P < 0.05$) (Table 2). Fetal weights were lowest in the marginal dietary folic acid–MeOH group. Mean fetal crown–rump lengths were similar among the groups. Fetal liver folate concentrations were significantly affected by maternal dietary folic acid intake. Fetal liver folate values were 63% lower ($P < 0.01$) in the marginal folic acid–water group, and 71% lower ($P < 0.01$) in the marginal folic acid–MeOH group, compared to their respective adequate folate controls (Table 2).

The influence of dietary folic acid and MeOH treatment on the incidence of fetal malformations is summarized in Table 3. The incidence of cleft palate was affected by both dietary folic acid and MeOH treatments. Dietary folic acid treatment independently affected the incidence of cleft palate; no cases were observed in the adequate folic acid–water group while 13.6% of the litters examined in the marginal folic acid–water group had at least one case of cleft palate. MeOH significantly el-

Table 2. The influence of dietary folic acid intake (400 and 1200 nmol folic acid/kg diet) on the effects of methanol on reproductive parameters

	Marginal folate		Adequate folate	
	Water	MeOH	Water	MeOH
Litters (n)	(22)	(22)	(24)	(21)
Implantation sites (#/litter)	11.96 ± 0.49[a]	12.36 ± 0.49[a]	12.42 ± 0.45[a]	13.05 ± 0.33[a]
Live fetuses (#/litter)	9.77 ± 0.54[b]	10.68 ± 0.42[a,b]	11.75 ± 0.44[a]	10.57 ± 0.54[a,b]
Live fetuses (%/litter)	82.03 ± 3.29[b]	86.86 ± 2.93[b]	94.59 ± 1.21[a]	80.82 ± 3.39[b]
Resorptions (%/litter)	16.92 ± 3.31[b]	11.02 ± 2.71[a,b]	5.41 ± 1.21[a]	13.99 ± 2.74[b]
Dead fetuses (%/litter)	1.02 ± 0.76[a]	2.10 ± 0.91[a]	0.0 ± 0.0[a]	5.17 ± 1.47[b]
Malformed fetuses (%/litter)	2.22 ± 1.42[a]	16.79 ± 3.63[b]	0.38 ± 0.38[a]	3.12 ± 1.29[a]
Total sites affected (#/litter)	2.50 ± 0.50[b]	3.82 ± 0.67[b]	0.71 ± 0.14[a]	2.86 ± 0.41[b]
Total sites affected (%litter)	20.20 ± 3.76[b]	29.95 ± 4.99[b]	5.85 ± 1.19[a]	22.3 ± 3.34[b]
Fetal weight (g)	1.12 ± 0.04[b]	1.07 ± 0.02[b]	1.26 ± 0.02[a]	1.12 ± 0.04[b]
Fetal crown–rump length (cm)	2.46 ± 0.04[a]	2.44 ± 0.03[a]	2.53 ± 0.02[a]	2.43 ± 0.06[a]
Fetal liver folate (nmol/g)	1.86 ± 0.15[b]	1.69 ± 0.12[b]	5.04 ± 0.22[a]	5.89 ± 0.39[a]

Methanol was given orally at a dose of 2.5 g/kg body weight, twice daily, between gestation days 6–10. Number in parentheses is the number of dams. Values are expressed as mean ± SEM, using litter as the unit of comparison. Values in a row with different superscript are significantly different ($P < 0.05$).

evated the occurrence of cleft palate in both dietary groups, with this effect being most pronounced in the marginal folic acid group, where 73% of the litters had at least one case. MeOH increased the incidence of fetuses affected by both cleft palate and exencephaly, with the highest incidence occurring in the marginal folic acid–MeOH-treated group. The overall frequency of fetal abnormalities was influenced by both folic acid and MeOH treatment, the highest frequency being in the marginal folic acid–MeOH group.

The frequency of MN in maternal and fetal reticulocytes (Table 4) was similar among the groups; the frequency of MN was not associated with either maternal or fetal hepatic folate concentrations. Table 5 shows that there was no significant difference in the frequency of MN in reticulocytes of fetuses that were affected by malformations compared to fetuses without malformations.

DISCUSSION

The marginal folic acid treatment used in our study substantially reduced the size of the pool of readily available folate as indicated by the low concentrations of plasma folate, as well as the size of the pool of folate stores, as reflected by the low erythrocyte and hepatic tissue folate concentrations. In pregnant Swiss–Webster mice, the consumption of a marginal folic acid diet similar to the one used in this study resulted in an even greater reduction in maternal folate indices (27). It is not clear if this difference is due to a differential response

Table 3. The influence of dietary folic acid intake (400 and 1200 nmol folic acid/kg diet) on the effects of methanol on fetal malformations

	Marginal folate		Adequate folate	
	Water	MeOH	Water	MeOH
Litters (n)	(22)	(22)	(24)	(21)
	(% litters affected)			
Fetal abnormalities	(Fetuses affected/total live fetuses)			
Cleft palate	13.6 ± 7.5[b]	72.7 ± 9.7[c]	0.0 ± 0[a]	19.0 ± 8.8[b]
	5/215	39/235	0/282	4/222
Excencephaly	13.6 ± 7.5[a,c]	22.7 ± 9.1[b,c]	4.2 ± 4.2[a]	19.0 ± 8.8[a,c]
	2/215	8/235	1/282	5/222
Combined cleft palate and excencephaly*	4.5 ± 4.5[b]	18.2 ± 8.4[c]	0.0 ± 0.0[a]	4.8 ± 4.8[b]
	1/215	4/235	0/282	1/222
Total affected fetuses	22.7 ± 9.1[b]	77.3 ± 9.1[c]	4.2 ± 4.2[a]	33.3 ± 10.5[b]
	6/215	43/235	1/282	8/222

Methanol was given orally at a dose of 2.5 g/kg body weight, twice daily, between gestation days 6–10. Number in parentheses is the number of dams. Values are expressed as mean ± SEM, using litter as the unit of comparison. Values in a row with different superscript are significantly different ($P < 0.05$).

*Fetuses characterized by having both defects.

Table 4. Effect of dietary folic acid intake (400 and 1200 nmol folic acid/kg diet) and methanol on the frequency of micronuclei in maternal and fetal reticulocytes

	Marginal folate		Adequate folate	
	Water	MeOH	Water	MeOH
Litters (n)	(9)	(14)	(12)	(13)
Maternal MN (per/1000 reticulocytes)	9.31 ± 1.83	9.56 ± 1.10	10.40 ± 1.40	9.64 ± 0.81
Litters (n)	(9)	(17)	(14)	(13)
Fetal MN (per/1000 reticulocytes)	9.52 ± 1.41	8.51 ± 0.97	7.39 ± 1.08	9.79 ± 0.96

Methanol was given orally at a dose of 2.5 g/kg body weight, twice daily, between gestation days 6–10. Number in parentheses is the number of dams. Values are expressed as Mean ± SEM; Values in a row were not significantly different ($P > 0.05$) by binomial coefficient statistics. MN = micronuclei.

of the two mouse strains to folate deficiency or if the baseline tissue folate concentrations in the two groups of animals were different at the initiation of pregnancy. Regardless of the reason, it is evident that the consumption of an amino acid-based diet containing 400 nmol folic acid/kg will result in a condition of marginal folate deficiency.

The reduced maternal folate status led to an appreciable reduction in the fetal liver folate concentrations. Significantly, this reduction exceeded that observed in the dams. It is important to note that the fetal folate stores were compromised in the absence of overt physical signs of maternal folate deficiency. Deriving from our results, we can speculate that this compromised fetal folate status would have an adverse effect on postnatal development. With respect to humans, this is a concern for the developing conceptus, given that marginal folate deficiency is prevalent in pregnant women (32). In rats, Thenen (33) has also reported that fetal liver folate concentrations were more affected than were maternal liver folate concentrations; however, in this study the diet that was fed was completely devoid of folate. Lin (34) has also reported that in rats, fetal liver folate concentrations can be influenced by maternal dietary folate intake. Collectively, these data suggest that the fetus does not have preferential access to maternal folate stores. It is interesting to note that in the reports by Thenen (33) and Lin (34), there was no discussion of the occurrence of cleft palate in their folate deficient groups. It is not clear if this reflects a difference in the response of rats and mice to the folate deficiency, or if it reflects other differences in the experimental design.

Previously, Rogers et al. (15) reported that giving CD-1 mice MeOH orally from GD 6 through 15 (4 g/kg/d) resulted in fetal growth retardation, an increased incidence of fetal malformations including cleft palate and exencephaly, and an increased frequency of prenatal death. The severity of the developmental toxicity with this oral dose of MeOH was similar to that observed in a 10,000 ppm inhalation exposure group (15). In a previous study, we reported that a 5 g/kg/d dose of MeOH produced a significant increase in the incidences of exencephaly and cleft palate, similar to the findings reported by Rogers et al. (15). The MeOH exposure paradigm in the present study was modified from our previous work in that dosing was from GD 6 through 10 instead of GD 6 through 15. Rogers et al. (35) have reported that the critical periods for MeOH-induced cleft palate and exencephaly fall within GD 6 through 10. Consistent with the above, we observed fetotoxic effects of MeOH that were similar to those in our previous report despite the shorter period of MeOH dosage.

Detoxification of MeOH occurs via a folate-dependent pathway; specifically, formate is converted to 10-formyl-THF, as catalyzed by 10-formyl-THF synthetase and the cosubstrate THF, followed by oxidation to carbon dioxide through the mediation of 10-formyl-THF dehydrogenase. Studies have demonstrated that the rate of formate oxidation is dependent upon the availability of hepatic folate stores (3,6,10). Our data indicate a trend toward lower plasma folate concentrations in both dietary groups with MeOH exposure. MeOH treatment decreased the plasma folate concentration in the adequate folic acid group to a value comparable to the plasma

Table 5. The frequency of micronuclei in fetal reticulocytes of normal and affected litters

	Marginal folate		Adequate folate	
	Water	MeOH	Water	MeOH
Normal litters (n)	(6)	(9)	(13)	(9)
Micronuclei (per/1000)	11.50 ± 1.46	6.09 ± 0.77	7.34 ± 1.17	9.79 ± 1.31
Affected litters (n)	(3)	(8)	(1)	(4)
Micronuclei (per/1000)	5.57 ± 1.23	11.22 ± 1.33	8.0	9.80 ± 1.29

Number in parentheses is the number of dams. Mean ± SEM; Values in a row were not significantly different ($P > 0.05$) by binomial coefficient statistics.

folate concentration of the marginal folic acid–water group. These data support the concept that oxidation of MeOH-derived formate may exert a stress on folate metabolism and affect the available folate pools in adequate folic acid-fed dams. Because adequate hepatic folate stores are critical for the formate oxidation process, it is possible that hepatic folate concentrations are maintained by decreasing the release of folate into blood, potentially explaining why an effect of MeOH on hepatic tissue folate concentrations was not observed. It is important to note that plasma folate concentrations in the present study were determined on GD 18, 8 d after the final MeOH exposure. Therefore, the effect of MeOH on the plasma folate pool was possibly more severe during the gavage period, supporting the concept that suboptimal folate transfer to the embryo could have occurred during the period of MeOH gavage. Dorman *et al.* (36) have reported that CD-1 mice exposed to high levels of MeOH through inhalation (> 10,000 ppm) on GD 8 develop a transient depletion of RBC folate concentrations within 2 h following the exposure. We are currently investigating the immediate effect of MeOH on maternal folate stores.

Consistent with previous reports (15,19,37), MeOH exposure in our study resulted in a higher frequency of cleft palate than exencephaly. Significantly, we also saw an independent effect of marginal dietary folic acid intake on the incidence of cleft palate. This finding of an effect of folic acid on the incidence of cleft palate is consistent with the recent epidemiologic studies that suggest that folic acid-containing multivitamin supplementation offer protection against the recurrence (38) and occurrence (39) of orofacial clefts. Additional studies are required to confirm the effect of dietary folic acid on the risk for cleft palate in this animal model.

To date, we are unaware of reports relating fetal liver folate concentrations with the occurrence of fetal abnormalities as a result of a specific deficiency of dietary folic acid. Ideally, we would measure the fetal hepatic folate concentration in individually affected fetuses to assess the relationship between fetal folate status and fetal abnormalities; however, the limited amount of fetal liver obtained from each fetuses did not permit us to do so in the current study. Instead, fetal livers were pooled from the litter for tissue folate analysis, regardless if the fetus was normal or malformed. However, with this caveat, we did not observe any difference in fetal folate stores between the affected and unaffected litters when comparing the values within the same dietary group.

Mechanistically, given the role of folate in pyrimidine and purine synthesis, we have proposed that a low folate status may lead to a nucleotide imbalance that would affect the rate of cell proliferation and differentiation. We hypothesized that the additional stress of MeOH detoxification occurring via a folate-dependent pathway would enhance cytogenetic damage in vivo by further altering folate metabolism. However, we did not find the measurement of MN to be a sensitive marker for either marginal folate deficiency or MeOH exposure. We have also measured the frequency of MN in maternal and fetal reticulocytes and found no evidence of increased cytogenetic damage with respect to fetal abnormalities.

In summary, the consumption of marginal folic acid diet resulted in low maternal tissue folate and fetal liver folate concentrations. MeOH acted synergistically with the compromised maternal and fetal folate status to adversely affect fetal development resulting in cleft palate. An additional significant finding of our study was that reduced fetal folate stores were shown to associate with an increased risk for cleft palate. No evidence of increased cytogenetic damage was found in the malformed litters, indicating that the assessment of MN is not of value as a biomarker for predicting the reproductive risk of MeOH in marginally folate deficient CD-1 mice. Given the accelerated catabolism of folate in women during early pregnancy (40), an inadequate dietary folate intake, or additional environmental stresses such as increased MeOH exposure may potentially induce transient folate deficiency in marginally folate deficient women. Our results warrant further investigations on the mechanisms of the developmental toxicity of MeOH and the environmental factors that can modulate its toxicity.

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REFERENCES

1. Kavet R, Nauss KM. The toxicity of inhaled methanol vapors. Crit Rev Toxicol. 1990;21:21–50.
2. Clay KL, Murphy RC, Watkins WD. Experimental methanol toxicity in the primates: analysis of metabolic acidosis. Toxicol Appl Pharmacol. 1975;34:49–61.
3. Eells JT. Methanol-induced visual toxicity in the rat. J Pharmacol Exp Ther. 1991;257:56–63.
4. Liesivuori J, Savolainen H. Methanol and formic acid toxicity: biochemical mechanisms. Pharmacol Toxicol. 1991;69:157–63.
5. Garner CD, Lee EW. Evaluation of methanol-induced retinotoxicity using oscillatory potential analysis. Toxicology. 1994;93:113–24.
6. Garner CD, Lee EW, Terzo TS, Louis–Ferdinand RT. Role of retinal metabolism in methanol-induced retinal toxicity. J Toxicol Environ Health. 1995;44:43–56.
7. Roe O. Species differences in methanol poisoning. CRC Crit Rev Toxicol. 1982;10:275–86.
8. Eells JT, Black KA, Makar AB, Tedford CE, Tephly TR. The regulation of one-carbon oxidation in the rat by nitrous oxide and methionine. Arch Biochem Biophys. 1982;219:316–26.
9. Makar AB, Tephly TR. Methanol poisoning in the folate-deficient rat. Nature. 1976;261:715–6.
10. Lee EW, Garner CD, Terzo TS. Animal model for the study of methanol toxicity: comparison of folate-reduced rat responses with published monkey data. J Toxicol Environ Health. 1994;41:71–82.
11. Tephly TR. The toxicity of methanol. Life Sci. 1991;48:1031–41.

12. Suit PF, Estes ML. Methanol intoxication: clinical features and differential diagnosis. Cleveland Clin J Med. 1990;57:464–71.
13. Nelson BK, Brightwell WS, MacKenzie DR, et al. Teratological assessment of methanol and ethanol at high inhalation levels in rats. Exp Appl Toxicol. 1985;5:727–36.
14. Infurna R, Weiss B. Neonatal behavioral toxicity in rats following prenatal exposure to methanol. Teratology. 1986;33:259–65.
15. Rogers JM, Mole ML, Chernoff N, et al. The developmental toxicity of inhaled methanol in the CD-1 mouse, with quantitative dose–response modeling for estimation of benchmark doses. Teratology. 1993;47:175–88.
16. Andrews JE, Ebron–McCoy M, Logsdon TR, Mole LM, Kavlock RJ, Rogers JM. Developmental toxicity of methanol in whole embryo culture: a comparative study with mouse and rat embryos. Toxicology. 1993;81:205–15.
17. Andrews JE, Ebron–McCoy M, Kavlock RJ, Rogers JM. Developmental toxicity of formate and formic acid in whole embryo culture: a comparative study with mouse and rat embryos. Teratology. 1995;51:243–51.
18. Brown–Woodman PDC, Webster WS, Huq F, Herlihy C, Hayes LC, Picker K. Induction of birth defects by exposure to solvents: an *in vitro* study. Teratology. 1995;51:288.
19. Sakanashi TM, Rogers JM, Keen CL. Influence of folic acid intake on the developmental toxicity of methanol in the CD-1 mouse. Teratology. 1994;49:368.
20. Heddle JA, Cimino MC, Hayashi M, et al. Micronuclei as an index of cytogenetic damage: past, present, and future. Environ Mol Mutagen. 1991;182:277–91.
21. Everson RB, Wehr CM, Erexson GL, MacGregor JT. Association of marginal folate depletion with increased human chromosomal damage in vivo: demonstration by analysis of micronucleated erythrocytes. J Natl Cancer Inst. 1988;80:525–9.
22. Fenech M, Rinaldi J. The relationship between micronuclei in human lymphocytes and plasma levels of vitamin C, vitamin E, vitamin B12 and folic acid. Carcinogenesis. 1994;15:1405–11.
23. MacGregor JT, Schlegel R, Wehr CM, Alperin P, Ames BN. Cytogenetic damage induced by folate deficiency in mice is enhanced by caffeine. Proc Natl Acad Sci USA. 1990;87:9962–5.
24. Walzem RL, Clifford AJ. Folate deficiency in rats fed diets containing free amino acids or intact proteins. J Nutr. 1988;118:1089–96.
25. Culling CFA. Handbook of histopathological and histochemical techniques. 3rd ed. London: Butterworth; 1974.
26. Wilson SD, Horne DW. High-performance liquid chromatographic determination of the distribution of naturally occurring folic acid derivatives in rat liver. Anal Biochem. 1984;142:529–35.
27. Heid MK, Bills ND, Hinrichs SH, Clifford AJ. Folate deficiency alone does not produce neural tube defects in mice. J Nutr. 1992; 122:888–94.
28. Wilson SD, Horne DW. Use of glycerol-cryoprotected Lactobacillus casei for microbiological assay of folic acid. Clin Chem. 1982; 28:1198–200.
29. Keagy PM. Folacin. Microbiological and animal assays. Methods of vitamin assay. 4th ed. New York: John Wiley & Sons; 1985: 445–71.
30. Horne DW, Patterson D. Lactobacillus casei microbiological assay of folic acid derivatives in 96-well microtiter plates. Clin Chem. 1988;34:2357–9.
31. Snedecor GW, Cochran WG. Statistical methods. 7th ed. Ames, IA: Iowa State University Press; 1977.
32. Senti FR, Pilch SM. Analysis of folate data from the second National Health and Nutrition Examination Survey (NHANES II). J Nutr. 1985;115:1398–402.
33. Thenen SW. Correlation between maternal and fetal folic acid status at day 21 of gestation in rats. Nutr Rep Int. 1979;19:267–74.
34. Lin GW-J. Effect of dietary folic acid levels and gestational ethanol consumption on tissue folate contents and rat fetal development. Nutr Res. 1991;11:223–30.
35. Rogers JM, Barbee BD, Rehnberg BF. Critical periods of sensitivity for the developmental toxicity of inhaled methanol. Teratology. 1993;47:395.
36. Dorman DC, Bolon B, Struve MF, et al. Role of formate in methanol-induced exencephaly in CD-1 mice. Teratology. 1995;52:30–40.
37. Bolon B, Dorman DC, Janszen D, Morgan KT, Welsch F. Phase-specific developmental toxicity in mice following maternal methanol inhalation. Fundam Appl Toxicol. 1993;21:508–16.
38. Tolarova M, Harris J. Reduced recurrence of orofacial clefts after periconceptional supplementation with high-dose folic acid and multivitamins. Teratology. 1995;51:71–8.
39. Shaw GM, Lammer EJ, Wasserman CR, O'Malley CD, Tolarova MM. Risks of orofacial clefts in children born to women using multivitamins containing folic acid periconceptionally. Lancet. 1995;345:393–6.
40. McPartlin J, Halligan A, Scott JM, Darling M, Weir DG. Accelerated folate breakdown in pregnancy. Lancet. 1993;341:148–9.