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Glutathione depletion modulates methanol, formaldehyde and formate toxicity in cultured rat conceptuses

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Abstract

The proposed use of methanol (H_3COH) as an alternative to fossil fuels has prompted concern about potential health risks resulting from widespread environmental exposure. Methanol is teratogenic in rodents and, although the exact toxic species is not known, teratogenesis may result from the enzymatic biotransformation of H_3COH to formaldehyde (CH_2O) and formic acid causing increased biological reactivity and toxicity. A protective role for the antioxidant glutathione (GSH) has been described for H_3COH, CH_2O and formic acid toxicity in various biological systems but has yet to be evaluated in the developing conceptus. Whole embryo culture studies were conducted using GD 10-11 rat conceptuses to elucidate the relationship between H_3COH and its metabolites and GSH status. Methanol exposure produced a decrease in normal growth parameters and a dose-dependent loss of viability. CH_2O had deleterious effects on embryo growth and viability. Sodium formate (HCOONa) exposure resulted in a high mortality rate but viable embryos did not manifest any abnormalities. Methanol, CH_2O, and HCOONa all produced a significant depletion of GSH in both embryo and VYS. Inhibition of GSH synthesis by L-buthionine-*S,R*-sulfoximine (BSO) treatment exacerbated H_3COH, CH_2O and HCOONa embryotoxicity. Interestingly, only H_3COH/BSO and CH_2O/BSO co-treatments caused increased malformation, while embryos treated with HCOONa/BSO did not produce any developmental deformities. These results implicate CH_2O as the most embryotoxic H_3COH metabolite, on a molar basis, in terms of causing dysmorphogenesis, alterations of normal growth parameters and embryolethality. HCOONa was selectively embryolethal and did not produce dysmorphogenesis. CH_2O toxicity is potentiated by GSH depletion, indicating that GSH may be more directly involved in its detoxication in the embryo.

Abbreviations: ADH1, alcohol dehydrogenase 1; ADH3, alcohol dehydrogenase 3; BSO, L-buthionine-*S,R*-sulfoximine; CH_2O, formaldehyde; DPTA, diethylenetriaminepentaacetic acid; FA, formic acid; GSH, glutathione; H_3COH, methanol; HBSS, Hank's balanced salt solution; HEPES, N-[2-hydroxyethyl]-piperazine-N′-[3-propane-sulfonic acid]; MSA, methanesulfonic acid; HCOONa, sodium formate; ROS, reactive oxygen species; VYS, visceral yolk sac

Introduction

Methanol (H_3COH) has a variety of uses, such as in agricultural, laboratory, industrial, and domestic settings primarily as solvent but is also present in cosmetic formulations (0.1% to 5% of the preparation (Food and Drug Administration, 1984), foods such as fruits, juices (140 mg/L), and vegetables (Lanigan, 2001) and fermented beverages (1.5 g/L) (Von Burg, 1994). Moreover, H_3COH may become even more commonplace for human exposure as it has been suggested as a fossil fuel alternative. As H_3COH becomes more frequently used by the public, understanding H_3COH developmental toxicity will become an increasingly important issue.

Methanol has been described to have detrimental effects on development in several animal models following exposure by either inhalation or oral routes. Among rodents, both rat and mouse embryos have been shown to be directly sensitive to H_3COH in whole embryo culture, although, mice are considerably more sensitive (Andrews et al., 1995). Inhalation exposure of H_3COH ($>20\,000$ ppm) to pregnant rats resulted numerous defects, including exencephaly, encephalocele and skeletal and visceral abnormalities (Nelson et al., 1985). Infurna and Weiss (1986) showed that methanol exposure via oral gavage causes similar types of effects in rats as were observed in inhalation studies, albeit not as severe, but also produced additional behavior abnormalities, such as the failure to suckle and home correctly. Even more serious developmental defects, such as cleft palate and exencephaly, were observed in CD-1 mice dosed orally with $\sim$4 mg/kg H_3COH during organogenesis, from GD 6–15 (Bolon et al., 1993). In rats, a single oral dosing of H_3COH resulted in the failure of testicles to descend properly, exopthalmia and anopthalmia, but did not produce any clinical manifestations in the dam (Youssef et al., 1997).

The toxicokinetics of H_3COH implicate the developing fetus as a sensitive target for H_3COH-induced toxicity. Ward and Pollack (1996) showed that pregnant rats and mice did not eliminate H_3COH as well as non-pregnant animals following a single bolus injection, only achieving approximately 60% of the non-pregnant dam elimination. In another study by the same group, pregnant dams were dosed with H_3COH via bolus injection. Embryos had approximately 25% greater H_3COH concentrations at distribution equilibrium than maternal tissues (Ward and Pollack, 1995). Due to excessive accumulation of H_3COH during pregnancy and preferential H_3COH buildup in the embryo proper, insight into mechanisms for H_3COH metabolism and elimination is paramount for providing protection against H_3COH-induced developmental toxicity.

In adults, the majority of H_3COH metabolism occurs in the liver, but an understanding how the conceptus metabolizes and detoxicates H_3COH is lacking. Generally, H_3COH is metabolized into formaldehyde (CH_2O) via alcohol dehydrogenase 1 (ADH1), the predominate enzyme in humans and non-human primates involved in the initial steps of H_3COH detoxication (Figure 1). It is also known that CH_2O can be formed from H_3COH through a hydroxyl radical-mediated pathway. Formaldehyde does not accumulate in tissues as it is rapidly converted by its reaction with glutathione (GSH) to form *S*-formylglutathione, which is mediated by the NAD-dependent enzyme, formaldehyde dehydrogenase or alcohol dehydrogenase 3 (ADH3). Thiolase converts *S*-formylglutathione to formic acid (FA), which is widely believed to be the species that results in various birth defects. The third and terminal reaction that converts formate to CO_2 and water is catalyzed primarily by catalase (Kavet and Nauss, 1990; Von Burg, 1994).

Methanol exposure in rats via intraperitoneal injection caused a significant GSH deple-

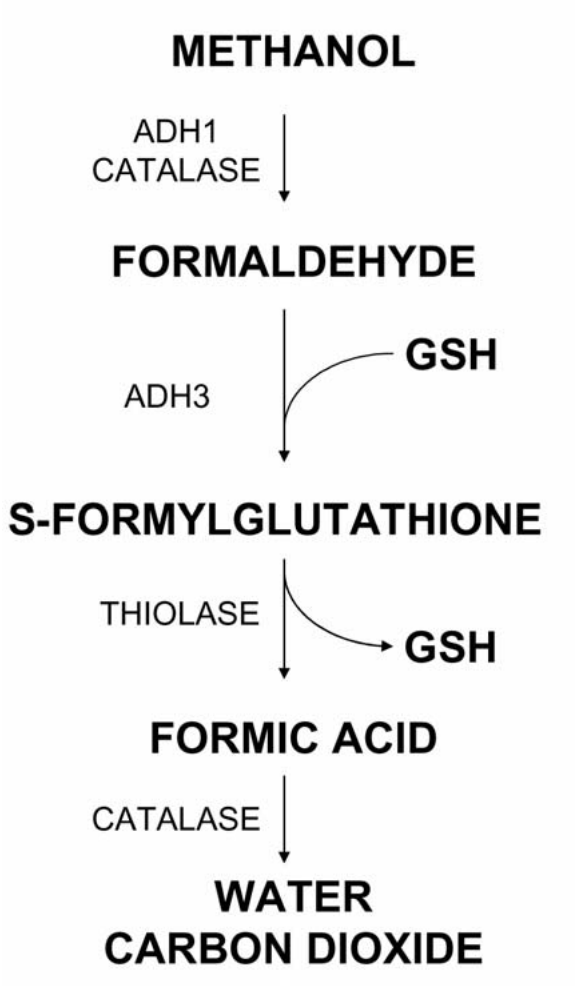

Figure 1. Methanol metabolism. Overview of the metabolism and the enzymes proposed to be involved in the conversion of methanol to water and CO_2.

tion in the brain after 2 days (~23%) and 7 days (~52%) (Farbiszewski et al., 2000). In the same study, lipid peroxidiation was determined using the thiobarbituric acid-reacting substances (TBARS) assay. The rat brain showed increased levels of lipid peroxidation after 2 days (increased by 331% of control) and 7 days (increased by 340% of control) with H_3COH exposure (Farbiszewski et al., 2000). Supplementation with antioxidants curbed both GSH depletion and lipid peroxidation. These findings suggest that H_3COH may, directly or indirectly, cause oxidative stress in the brain and may also be present in other tissues as well, and implies an even greater role for antioxidants, like GSH.

Although GSH is an integral part of the H_3COH detoxication pathway, its role has not been well-defined following H_3COH exposure in respect to the developing conceptus. In the present study, we utilized whole embryo culture to evaluate the effect of H_3COH and its metabolites (CH_2O and FA) on gross developmental parameters under conditions where GSH is depleted. These experiments may provide a greater understanding of how GSH contributes to H_3COH detoxication in the embryo and identify which chemical species may be most responsible for teratogenesis and embryolethality.

Materials and methods

Chemicals

Methanol, formaldehyde, sodium formate, acetonitrile, methanesulfonic acid (MSA), diethylenetriaminepentaacetic acid (DPTA), N-[2-hydroxyethyl]-piperazine-N′-[3-propanesulfonic acid] (HEPES) and L-buthionine-*S,R*-sulfoximine (BSO) were obtained from Sigma Chemical Co. (St Louis, MO). Monobromobimane (thiolyte) was obtained from Calbiochem (La Jolla, CA). Hank's balanced salt solution (HBSS) was acquired from Life Sciences (Grand Island, NY). Sodium methaneulfonate was purchased from Aldrich Chemical (Milwaukee, WI). All other reagents were obtained from common commercial sources.

Animals

Primagravida Sprague-Dawley rats were obtained from the Reproductive Sciences Program Small Animal Core, University of Michigan on GD 6–9. Day 0 was determined by a sperm-positive vaginal smear on the morning following copulation. Pregnant rats were maintained on a 12-h light/12-h dark cycle until explantation on GD 10. Food and water were given *ad libitum*.

Whole embryo culture

Rat whole embryo culture was performed as described by Fantel and colleagues (1989) and Harris and colleagues (1987). Each rat conceptus (8–10 somites) was prepared for culture on GD 10 and placed in bottles (1 conceptus/ml media) with warm media consisting of 33% (v/v) heat-inactivated pregnant rat serum and 66% HBSS (pH 7.4). Bottles containing conceptuses and media were placed in a rotating drum (BTC Engineering, Cambridge, UK) under constant gassing conditions of 20% O_2/5% CO_2/75% N_2 (v/v/v) and maintained at 37°C for a 20 h period. Atmospheric conditions were changed to 95% O_2/5% CO_2 (v/v) during the final 4 h on the following day (GD 11) to meet the metabolic requirements of the developing conceptus. Conceptuses were exposed to varying concentrations of H_3COH (12 and 24 mg/ml), CH_2O (3 and 5 μg/ ml) and HCOONa (0.5 and 2 mg/ml). Dose ranges were initially chosen based on other studies in whole embryo culture that produced optimal dysmorphogenesis while retaining a reasonably high viability (Andrews et al., 1995). Since very few H_3COH studies have centered on CH_2O, concentrations were determined by the generation of separate dose-response profiles (data not shown). In some cultures, exposure to H_3COH, CH_2O or HCOONa was preceded by prior treatment with BSO for 4 h. After termination of culture, growth developmental parameters were evaluated, including viability, embryonic rotation, neuropore closure, crown-rump length and somite formation. In addition, the visceral yolk sac (VYS) and embryo from H_3COH (24 mg/ml), CH_2O (3 μg/ml) and HCOONa (2 mg/ml) treatment groups (both with and without BSO pretreatments) were each placed in separate microcentrifuge tubes containing 200 μl of 200 mmol/L MSA for GSH and cysteine. Protein analysis was performed by the method of Bradford (Bradford, 1976). Measurements of GSH in embryos treated with 3 μg CH_2O/ml were analyzed rather than 6 μg/ml due to the increased toxicity at the higher CH_2O concentration. Samples were snap-frozen in liquid nitrogen and placed in a freezer at –70°C until prepared for HPLC analysis.

HPLC analysis

GSH and cysteine concentrations were measured using the HPLC method as described by Fahey and Newton (1987) and as modified by Harris and colleagues (1991). Frozen tissue samples were thawed and homogenized by ultrasonic disruption. Two hundred microliters of 4 mol/L sodium methanesulfonate to complete protein precipitation were added to each sample followed by centrifugation at 13 900 rpm for 5 min. The resulting supernatants were removed and separately placed into new microcentrifuge tubes containing 160 μl of 5 mmol/L DPTA in 1 mol/L Hepes (pH 8.5). Sample protein content was determined by solubilization of the pellet in 0.25 mol/L NaOH and was followed by protein assay as described by Bradford (1976). Derivatization of the supernatant was carried out by the addition of 20 μl of 0.2 mmol/L monobromobimane in acetonitrile. Samples were allowed to incubate for 20 min in the dark at room temperature. Derivatization was completed by the addition of 380 μl of 400 mmol/L MSA after which samples were snap-frozen in liquid nitrogen and stored at –70°C.

HPLC analyses were carried out by using a Waters NovaPak C 4-μm Radial-Pak cartridge fitted with a NovaPak Guard-Pak guard precolumn. Samples were eluted using an isocratic mobile phase consisting of 14.2% methanol (v/v) and 2.5% glacial acetic acid (v/v) at a flow rate of 1.0 ml/min. Following elution of both the cysteine and GSH peaks, the column was washed with 90% methanol (v/v) and 2.5% glacial acetic acid (v/v) for 15 min. Detection and quantitation of GSH-bimane and cysteine-bimane was accomplished using a Waters Model 470 scanning fluorescence detector (λ excitation 360 nm; λ emission 455 nm) followed by analysis and quantitation by a Waters Model 746 data module. Authentic standards were prepared and used to identify each peak of interest. These same standards were also used to quantify each sample. This method is sensitive enough to accurately detect GSH and cysteine levels as low as 10 pmol/injection.

Statistics

Statistical analyses were performed using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Viability, rotation and neuropore closure significant differences were determined by a z-test comparison of proportions of normal vs. abnormal parameters. Crown-rump length, somite number, GSH concentrations and cysteine concentrations significant differences were determined by one-way ANOVA followed by a Tukey's posthoc test. Significant differences were established where $p < 0.05$.

Results

Growth and developmental parameters

Methanol exposure. The addition of H_3COH into the culture medium resulted in significant effects on one or more of the growth parameters. The results are presented in Table 1. At 12 mg/ml and 24 mg/ml H_3COH, embryo viability and neuropore closure were significantly decreased. Similar trends were represented in the crown-rump measurement, where overall length was significantly decreased in all H_3COH groups. Somite number decreased with all H_3COH treatments and followed in a dose-dependent manner. Morphological observations of the conceptus included embryonic bloody blisters in treated embryos.

Formaldehyde exposure. Formaldehyde treatment (3 and 6 μg/ml) yielded various effects on embryo development, which are tabulated in Table 2. Treatment with 3 and 6 μg CH_2O/ml caused a significant decrease in embryo viability and rotation. Neuropore closure, crown-rump length and somite number measurements were unaffected at 3 μg/ml but were significantly different at 6 μg/ml. At the lower dose (3 μg/ml), some blister formation was observed. At a dose of 6 μg/ml, a greater number of embryos showed evidence of dysmorhphogenesis, including blisters and more extensive regions of apparent necrosis, identified by markedly increased tissue opacity.

Formate exposure. Sodium formate had little effects on growth and developmental parameters in surviving embryos as shown in Table 3. Embryonic viability was greatly affected with HCOONa treatments, but at the lower dose (0.5 mg/ml), the surviving embryos showed no developmental or growth dysmorphogenesis. Furthermore, in the higher dose (2 mg/ml), the only parameter that showed a significant change was somite number. One embryo at 0.5 mg/ml had blisters, and one embryo at 2 mg/ml appeared necrotic.

BSO co-treatments. Buthionine-*S,R*-sulfoximine treatment alone had no significant effects on most of the growth and developmental parameters. The only parameter affected was crown-rump length, where BSO treated embryos were slightly, but significantly, smaller (Tables 1–3).

Table 1. Treatment of GD 10-11 rat conceptuses with methanol (12–24 mg/ml) with or without BSO for 24 h *in vitro*.

Treatment	% Viability	% Rotation	% Closure	Length (mm) (n)	Somites (n)	Malformations (n)
Control	100 (30/30)	97 (29/30)	93 (27/29)	3.4 ± 0.0 (29)	25.5 ± 0.3 (22)	None
H_3COH (12 mg/ml)	77* (27/35)	96 (26/27)	77* (20/26)	3.1 ± 0.0* (26)	22.1 ± 0.5* (27)	Blisters (4)
H_3COH (24 mg/ml)	60* (12/20)	100 (12/12)	50* (6/12)	2.7 ± 0.2* (12)	19.0 ± 0.0* (12)	Blisters (3) Necrotic (7)
BSO (2 mg/ml)	96 (27/28)	96 (26/27)	96 (25/26)	3.2 ± 0.0* (27)	24.8 ± 0.3 (26)	None
H_3COH (12 mg/ml) BSO (2 mg/ml)	81 (13/16)	54** (7/13)	54 (7/13)	2.4 ± 0.1** (7)	19.0 ± 0.1** (13)	None
H_3COH (24 mg/ml) BSO (2 mg/ml)	50 (5/10)	20** (1/5)	0** (0/5)	2.0 ± 0.0** (5)	NA	Blisters (5)

*Significant difference from control ($p < 0.05$).

**Significant difference from H_3COH only ($p < 0.05$).

Table 2. Treatment of GD 10–11 rat conceptuses with formaldehyde (3–6 μg/ml) with or without BSO for 24 h *in vitro*

Treatment	% Viability	% Rotation	% Closure	Length (mm) (n)	Somites (n)	Malformations (n)
Control	100 (26/26)	96 (25/26)	96 (24/25)	3.4 ± 0.1 (25)	25.8 ± 0.3 (27)	None
CH_2O (3 μg /ml)	84* (21/25)	81* (17/21)	96 (19/21)	2.9 ± 0.1 (20)	24.7 ± 0.2 (18)	Blisters
CH_2O (6 μg/ml)	60* (12/20)	50* (6/12)	42* (5/12)	2.7 ± 0.1* (12)	20 ± 1.1 (12)	Blisters (10)
BSO (2 mg/ml)	92 (24/26)	95 (23/24)	96 (22/23)	3.2 ± 0.0* (24)	24.9 ± 0.3 (21)	None
CH_2O (3 μg/ml) BSO (2 mg/ml)	40** (10/25)	20** (2/10)	20** (2/10)	2.2 ± 0.1 (2)	22.7 ± 0.8** (10)	Blisters (3) Necrotic (6)
CH_2O (6 μg/ml) BSO (2 mg/ml)	0** (0/18)	NA	NA	NA	NA	NA

Significant difference from control ($p < 0.05$).

**Significant difference from CH_2O only ($p < 0.05$).

Table 3. Treatment of GD 10–11 rat conceptuses with sodium formate (0.5–2 mg/ml) with or without BSO for 24 h *in vitro*

Treatment	% Viability	% Rotation	% Closure	Length (mm) (n)	Somites (n)	Malformations (n)
Control	100 (26/26)	100 (26/26)	92 (22/24)	3.4 ± 0.0 (26)	25.5 ± 0.3 (26)	None
HCOONa (0.5 mg/ml)	84* (16/19)	100 (16/16)	94 (15/16)	3.4 ± 0.0 (16)	24.7 ± 0.2 (18)	Blisters (1)
HCOONa (2 mg/ml)	40* (6/15)	100 (6/6)	100 (6/6)	3.4 ± 0.1 (6)	20 ± 1.1* (12)	Necrotic (2)
BSO (2 mg/ml)	95 (21/22)	95 (20/21)	95 (19/20)	3.2 ± 0.1* (20)	25.1 ± 0.3 (20)	Blisters (1)
BSO (2 mg/ml) HCOONa (0.5 mg/ml)	71 (12/17)	83 (10/12)	100 (12/12)	3.2 ± 0.4 (10)	22.7 ± 0.8** (10)	Blisters (1) Necrotic (5)
BSO (2 mg/ml) HCOONa (2 mg/ml)	27** (4/15)	100 (4/4)	100 (4/4)	3.1 ± 0.4 (4)	NA	Necrotic

*Significant difference from control ($p < 0.05$).

**Significant difference from HCOONa only ($p < 0.05$).

At the lower dose (12 mg/ml), H_3COH co-treatment with BSO did not significantly decrease viability and neuropore closure as compared to conceptuses that received H_3COH only (Table 1). Embryos treated at this dose did not manifest any blister formation or appear necrotic. At 24 mg/ml H_3COH and BSO, embryonic rotation, neuropore and crown-rump length were decreased to a greater extent than conceptuses receiving H_3COH only. Somite formation was undetectable in H_3COH (24 mg/ml) and BSO treated embryos, and blister formation was evident.

Formaldehyde treatment of BSO-pretreated conceptuses caused significant changes to viability and development (Table 2). BSO pretreatment caused a significant decrease in viability, rotation, neuropore closure and somite number from conceptuses receiving H_2CO only (3 μg/ml). Blisters were observed in some embryos and a high level of necrosis in others. At the higher dose (6 μg/ml), BSO pretreatments cause complete death after the culture period.

Co-treatment with HCOONa (0.5 mg/ml) and BSO only affected somite number which was significantly different from embryos receiving HCOONa only (Table 3). At the higher concentration of HCOONa, treatments exacerbated the lethal effects but had no other effects on surviving embryos' growth parameters and appeared similar to control embryos.

Glutathione and cysteine

Control embryos contained approximately 14.0 (±2.41; $n = 32$) pmoles GSH/μg protein and control VYS contained 32.6 (±3.86; $n = 30$) pmoles GSH/μg protein (Figure 2). Buthionine sulfoximine treatment alone produced a significant GSH depletion after 24 h in

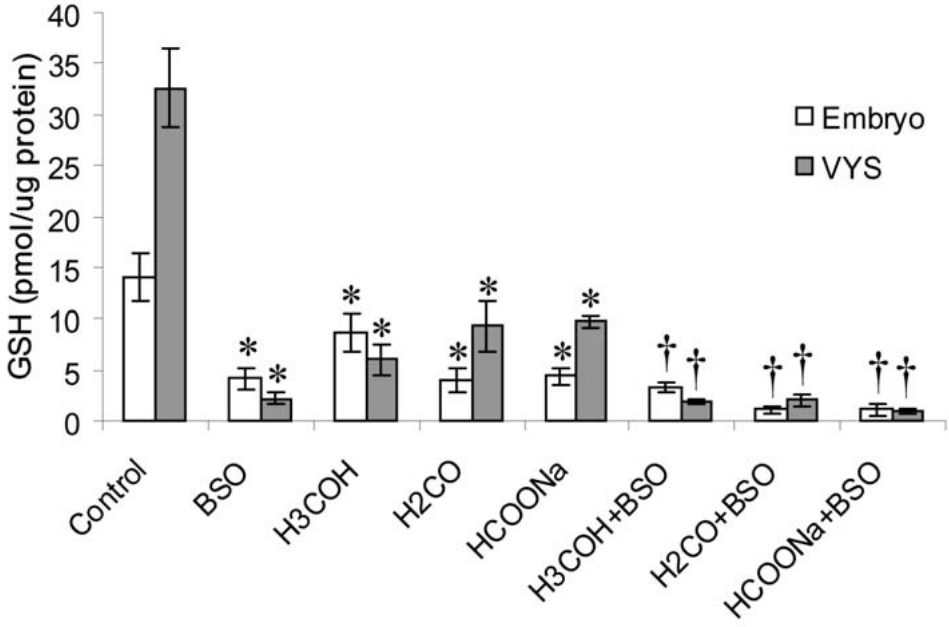

Figure 2. Embryonic glutathione concentrations in GD 11 rat embryos and visceral yolk sacs following buthionine-sulfoximine (BSO), H_3COH, CH_2O, HCOONa and H_3COH+BSO, CH_2O+BSO and HCOONa+BSO in culture for 24 h. Asterisks (*) denote a statistically significant difference ($p < 0.05$) between control. Crosses (†) denote a statistically significant difference ($p < 0.05$) between treatments with and without BSO.

both the embryo and VYS as compared to control conceptuses, measuring 4.1 ($\pm$1.02) and 3.3 ($\pm$0.53) pmoles GSH/μg protein, respectively (Figure 2).

Treatment with 24 mg/ml H_3COH resulted in a significant decrease ($p < 0.05$) in embryonic GSH concentrations by 38% (8.7 pmoles GSH/μg protein ($\pm$1.80; n = 26)), but at the same H_3COH concentrations, GSH was depleted to an even greater extent in the VYS by 83% (6.0 pmoles GSH/μg protein ($\pm$1.46; n = 14)). Treatment with both 24 mg H_3COH/ml and BSO caused an even greater GSH depletion that reached significance in both the embryo proper (3.3 pmoles GSH/μg protein ($\pm$0.54; n = 24)) and the VYS (1.9 pmoles GSH/μg protein ($\pm$0.22; n = 24)).

Treatment with CH_2O (3 μg/ml) caused GSH depletion, measuring 4.0 ($\pm$1.12; n = 12) and 9.3 ($\pm$2.41; n = 12) pmoles GSH/μg protein in the embryo and VYS respectively (Figure 2). Formaldehyde and BSO treatments increased the extent of GSH depletion in both the embryo (1.1 pmoles GSH/μg protein ($\pm$0.22; n = 11)) and VYS (2.0 pmoles GSH/μg protein ($\pm$0.31; n = 11)).

Finally, treatment with HCOONa depleted GSH as well the extent of which was very similar to CH_2O treated conceptuses, decreasing GSH in the embryo to 4.3 ($\pm$0.9; n = 6) pmoles GSH/μg protein and in the VYS to 9.8 ($\pm$0.61; n = 6) pmoles GSH/μg protein (Figure 2). Prior treatment with BSO further diminished GSH stores with HCOONa in both the embryo proper (1.1 ($\pm$0.6; n = 8) pmoles GSH/ μg protein) and the VYS (0.9 ($\pm$0.22; n = 8)).

Cysteine was concentrations were also determined. Control VYS contained 5.5 ($\pm$1.50; n = 32) pmoles cysteine/μg protein while control

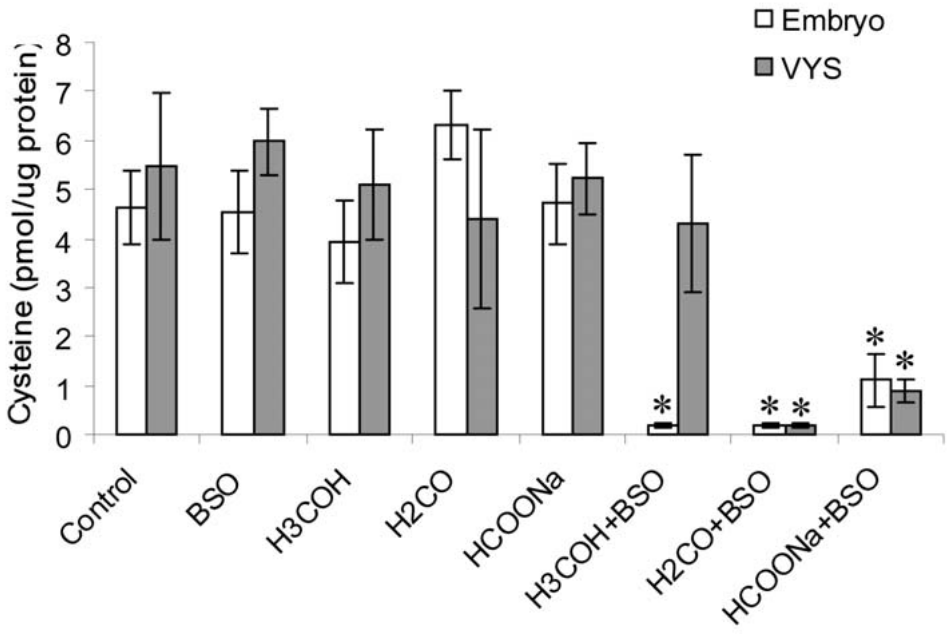

Figure 3. Cysteine concentrations in GD rat embryos and visceral yolk sacs following treatment with H_3COH, CH_2O or HCOONa with or without BSO treatment in culture for 24 h. Asterisks (*) denote a statistically significant difference ($p < 0.05$) between treatments with and without BSO.

embryos contained 4.6 ($\pm$0.74; n = 30) pmoles cysteine/μg protein (Figure 3). Conceptuses that received BSO only did not exhibit a significant decrease in cysteine content.

Methanol treatment (24 mg/ml) did not have a significant effect on cysteine concentrations in either the embryo proper or VYS (Figure 3). However, with prior BSO treatment, H_3COH exposure caused a significant cysteine depletion but only in the embryo (0.2 ($\pm$0.05; n = 26)) pmoles cysteine/μg protein) and not in the VYS (4.3 ($\pm$1.40; n = 14) pmoles cysteine/μg protein).

CH_2O treatment (3 μg/ml) increased cysteine in the embryo proper (6.3 ($\pm$0.07; n = 12) pmoles cysteine/μg protein) but the VYS remained relatively unchanged, measuring 4.4 ($\pm$1.81; n = 12) pmoles cysteine/μg protein (Figure 3). BSO and CH_2O co-treatments resulted in a reduction of cysteine pools in both the embryo (0.2 ($\pm$0.05; n = 11) pmoles cysteine/μg protein) and VYS (0.2 ($\pm$0.04; n = 11) pmoles cysteine/μg protein).

HCOONa exposure (2 mg/ml) had little effect in the embryo proper or in the VYS (Figure 3). With both BSO and HCOONa treatments, cysteine concentrations were significantly reduced in both the embryo (1.1 ($\pm$0.55; n = 8) pmoles cysteine/μg protein) and VYS (0.9 ($\pm$0.24; n = 8) pmoles cysteine/μg protein).

Discussion

Previous work to describe H_3COH and formate toxicity has been performed in rat whole embryo culture and produced similar growth and developmental abnormalities as observed in this study (Andrews et al., 1998). In both rat and mouse embryos, formic acid and HCOONa exposure *in vitro* resulted in the

inhibition of anterior and posterior neuropore closure, rotational defects, tail anomalies, enlarged pericardium and delayed heart development (Andrews et al., 1995). In our study, neuropore closure was inhibited by H_3COH and suggests the initiation of early events that lead to exencephaly, a common defect seen in rodents exposed to H_3COH via inhalation (Nelson et al., 1985; Bolon et al., 1993). In spite of limitations for whole embryo culture, where neural development cannot be followed to term *in vitro* to assess permanence of embryonic lesions, the common nature of malformations seen in whole embryo culture and data from *in vivo* H_3COH exposures help to validate the extrapolation to *in vivo* models. Furthermore, our results correlated well with these previous studies which utilized both H_3COH and formate, but have produced new data suggesting that CH_2O also contributes to a decrease in conceptus viability. Dysmorphogenesis and other developmental abnormalities are more prevalent in conceptuses treated with H_3COH and CH_2O while HCOONa treatments produced less dysmorphogenic effects and are selectively embryolethal. These data show that H_3COH and CH_2O, which rely on GSH for detoxication and elimination, are the most dysmorphogenic but that HCOONa, a post-GSH conjugation and elimination metabolite, produces the greatest lethality but no dysmorphogenesis. These results implicate GSH as a critical factor in H_3COH-induced dysmorphogenesis. The lack of dysmorphogenesis seen with HCOONa in the present study does not rule out the possibility of producing malformations but may simply reflect a choice of doses that bracket a very steep dose response.

Depletion of GSH via BSO alone did not effect embryonic development, but subsequent treatment with H_3COH caused significant developmental abnormalities and decreased viability in a dose-dependent manner. Formaldehyde had a similar effect on development. Although HCOONa proved to be very toxic, the remaining embryos did not manifest any malformations or altered growth parameters.

While not evaluated specifically in embryos, other studies have shown that H_3COH exposure in adult rats alters antioxidant enzymes, such as glutathione peroxidase and glutathione disulfide reductase, and antioxidants, such as GSH and ascorbate, in the liver, erythrocytes, brain, and blood serum (Skrzydlewska and Farbiszewski, 1998; Skrzydlewska et al., 1998). Glutathione plays a crucial role in the detoxication of ROS and reactive intermediates. Our study shows that H_3COH, CH_2O and HCOONa, at embryotoxic doses, all produce a significant decrease in GSH content in both the embryo and VYS, suggestive that GSH is active in the detoxication of H_3COH and/or H_3COH-related metabolites.

Another interesting observation is that H_3COH caused a greater depletion of GSH in the VYS than the embryo, while both CH_2O and HCOONa showed greater GSH depletion in the embryo proper. Preferential depletion by H_3COH of VYS GSH suggests that principle site of H_3COH detoxication is in this extra-embryonic organ, which may play a role as a primary line of defense to protect the embryo proper. Unlike human fetal development, the rat embryo is encompassed by the VYS throughout gestation and has approximately 65% more GSH ($\sim$20 pmoles/μg protein) than the embryo proper ($\sim$12 pmoles/μg protein) (Figure 2) (Hansen et al., 1999). Unlike H_3COH, CH_2O and HCOONa both caused a more substantial GSH depletion in the embryo, indicating that both CH_2O and HCOONa metabolism involving GSH may occur primarily in the embryo rather than the VYS. It should also be considered that a greater apparent GSH depletion occurs in the embryo due to a reduced capacity to supply precursor amino acids for new synthesis or due to an inability to enzymatically reduce oxidized GSH.

Prior depletion of GSH with BSO followed by subsequent treatments with H_3COH, CH_2O or HCOONa potentiated GSH depletion as compared to BSO alone and correlated with decreased viability. Growth and developmental parameters were only affected in conceptuses that received H_3COH and CH_2O but, interestingly, not HCOONa. These data suggest that HCOONa-mediated effects, namely decreased viability, are most likely not mediated by GSH and could be attributed more to acidosis as previously suggested by other studies (Lanigan, 2001). Decreased GSH in HCOONa-treated embryos may be a result of oxidation due to an acidic pH rather than active detoxication of HCOONa.

Methanol is converted to CH_2O by ADH1, located primarily in the liver of adults. However, rat embryonic livers (GD 12–20) have been shown to have very low alcohol dehydrogenase activity (Zorzana and Herrera, 1989) and may not be capable of metabolizing H_3COH, relying on other tissues and/or other methods to metabolize H_3COH. An alternative H_3COH metabolism pathway is through catalase, producing CH_2O and hydrogen peroxide (Lanigan, 2001). Previous work in our laboratory showed that rat embryos have 15–25% less ADH1 and ADH3 specific activity than the VYS during early organogenesis but rat embryo catalase activity is considerably higher than the VYS (Harris et al., 2003), implying that the embryo could better utilize the catalase pathway for H_3COH conversion to CH_2O. If the catalase detoxication pathway of H_3COH is primarily utilized by the embryo, it would offer some rationale to GSH depletion through hydrogen peroxide generation, unlike ADH1 metabolism where hydrogen peroxide is not generated. Furthermore, it provides additional evidence that the embryo is a sensitive target for H_3COH toxicity. An increased generation of hydrogen peroxide as a component of the mechanism of H_3COH embryotoxicity could also be important in explaining the clear and blood-filled blisters seen in exposed embryos. Generation of reactive oxygen species can produce large regions of O_2 depletion and the resulting moderate hypoxia is known to elicit these types of blistering effects in developing embryos.

Formaldehyde exposure had significant effects on viability and development, and these effects were potentiated by GSH depletion with BSO. Formaldehyde affected embryos at a concentration that was considerably lower than H_3COH ($\sim$100–200 μmol/L CH_2O vs $\sim$375–750 mmol/L H_3COH), suggesting teratogenesis may be a result of CH_2O rather than H_3COH. Formaldehyde, the main metabolite of H_3COH, relies largely on GSH for detoxication in rat conceptuses as viability declined and development was disrupted. The conversion of CH_2O to formate is dependent on GSH, as noted in the introduction (Figure 1), and GSH depletion by CH_2O alone may be due to its reactive nature. Decreased concentrations of GSH would block normal CH_2O elimination. The inability to form *S*-formylglutathione and the decrease of CH_2O's subsequent metabolism to formic acid may result in the accumulation of CH_2O and cause cell damage and death, eventually contributing to teratogenesis and other undesirable developmental outcomes.

Cysteine is of related interest as it is the rate-limiting precursor for GSH in *de novo* synthesis. Intriguingly, cysteine concentrations were relatively unaffected with H_3COH, CH_2O, or HCOONa treatments, indicating that although H_3COH, CH_2O, or HCOONa alone are capable of depleting GSH stores, both the embryo and VYS are capable of maintaining sufficient cysteine for *de novo* GSH synthesis. However, with BSO treatment, GSH synthesis is inhibited. Over time, GSH is lost through normal cellular turnover and may affect other reducing equivalents in the cell, such as cysteine, which, besides GSH, is one of the largest non-protein thiol pools in most cells. In each treatment

coupled with BSO, the already low embryonic cysteine stores may undergo oxidation to cystine, producing the dramatic pattern of cysteine loss seen in Figure 5. Very little is known about cysteine supply and regulation in the developing conceptus. Our previous studies show that the major source of cysteine to the embryo is through proteolytic degradation of maternal proteins in the VYS. It was speculated that restoration of depleted GSH in the embryo proper was dependent on GSH transported from the VYS in order to supply needed cysteine for new GSH synthesis (Harris, 1993). The intriguing observation that H_3COH, H2CO, and HCOONa alone do not affect cysteine levels but result in tissue selective depletions in combination with BSO cannot be explained based on our current knowledge. Cysteine/cystine transport is known to be inducible through insults that elicit oxidative stress and is sensitive to changes in oxygen concentrations (Sato et al., 2001). It is possible that combinations of BSO, H_3COH and its metabolites are producing conditions of hypoxia under which the transport of cystine is inhibited and to which the embryo may be selectively sensitive in comparison to the VYS.

Methanol, formaldehyde and formic acid are embryolethal, and methanol and formaldehyde are dysmorphogenic. However, mechanisms by which these compounds are detoxicated in the early organogenesis stage embryo are not completely understood. Glutathione is an important factor in methanol detoxication as it has been implicated in methanol metabolism in the adult liver. Here, we show evidence that glutathione is equally important in the detoxication of methanol and formaldehyde in the developing conceptus. Moreover, these findings suggest that during periods of GSH depletion, formaldehyde most likely accounts for the teratogenic outcomes observed in cultured embryos and formate treatment did not exacerbate malformation but only death.

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