Developmental Toxicity Interactions of Methanol and Radiofrequency Radiation or 2-Methoxyethanol in Rats

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This research was undertaken to determine potential interactions among chemical and physical agents. Radiofrequency (RF) radiation is used in numerous workplaces, and many workers are concurrently exposed to RF radiation and various chemicals. The developmental toxicity of RF radiation is associated with the degree and duration of hyperthermia induced by the exposure. Previous animal research indicates that hyperthermia induced by an elevation in ambient temperature can potentiate the toxicity and teratogenicity of some chemical agents. We previously demonstrated that combined exposure to RF radiation (10 MHz) and the industrial solvent, 2-methoxyethanol (2ME), enhanced teratogenicity in rats. Interactions were noted at even the lowest levels of 2ME tested, but only at hyperthermic levels of RF radiation. The purpose of the present research is to investigate if the interactive effects noted for RF radiation and 2ME are unique to these agents, or if similar interactions might be seen with other chemicals. Because methanol is widely used as a solvent as well as fuel additive, and, at high levels, is teratogenic in animals, we selected methanol as a chemical to address generalizability. Based on the literature and our pilot studies, 0, 2, or 3 g/kg methanol (twice, at 6-hour intervals) were administered on gestation day 9 or 13 to groups of 10 Sprague-Dawley rats. Dams treated on day 9 were given methanol and exposed to RF radiation sufficient to maintain colonic temperature at 41°C for 60 minutes (or sham). Those treated on day 13 were given methanol plus either 0 or 100 mg/kg 2ME. Because we observed that methanol produced hypothermia, some groups were given the initial dose of methanol concurrently with the RF or 2ME, and others were given the first dose of methanol 1.5 hours prior to RF or 2ME. Dams were sacrificed on gestation day 20, and the fetuses were examined for external malformations. The results indicate that RF radiation or methanol on day 9 increased the incidence of resorbed fetuses, but no interactive effects were observed. The resorptions were highest in groups given the experimental treatments 1.5 hours apart. The higher dose of methanol also reduced fetal weights. Administration of 2ME or methanol on day 13 increased the rate of malformations, and there was evidence of a positive interaction between 2ME and methanol. Fetal weights were reduced by 2ME and methanol alone, but no interaction was observed. Also, separation of the dosing with the teratogens did not affect the results. These results point out that interactions in developmental toxicology, such as those of RF radiation, 2ME, and methanol that we have studied, are complex, and such interactions cannot be fully understood or predicted without more research. It is important that combined exposure effects be considered when developing both physical agent and chemical agent exposure guidelines and intervention strategies.

Keywords Developmental Toxicity, Exposure Standards, Glycol Ethers, Hyperthermia, Industrial Solvents, Intervention Strategies, Methanol, RF Radiation, Synergism

Radiofrequency (RF) radiation is used in numerous workplaces (Fox, Knadle, and Brook 1976; Conover et al. 1981; Cox, Murray, and Foley 1982; Cohen 1986). In addition to RF radiation, many workers can be concurrently exposed to various chemicals—some of which could be teratogenic at certain levels (Goldstein et al. 1976; Cox and Conover 1981; Stewart and Elkington 1985; Cone 1986; Schardein 1993). Concurrent exposures to various agents can sometimes produce developmental toxicity that is very different from that seen with individual exposures (e.g., Fraser 1977; Goldstein, Hewitt, and Hook 1990; Nelson 1994, 1997).

Isolated reports suggest that RF radiation may be developmentally toxic in humans (e.g., Marchese 1953; Rubin and Erdman 1959; Cocozza, De Blasio, and Nunziata 1960; Hoffman and Dietzel 1966; Imrie 1971; Brown-Woodman et al. 1988), although we do not believe that occupational exposures would normally produce these effects. In experimental animals, the teratogenicity of RF radiation is associated with the degree and duration of hyperthermia it produces (e.g., Chernovitz, Justesen, and Oke 1977; Nawrot, McRee, and Staples 1981; Lary et al. 1982, 1983, 1986; Lary and Conover 1987). Hyperthermia produces congenital malformations in experimental animals, and it has been hypothesized to produce malformations in humans (Edwards 1986; Lary 1986; Warkany 1986; Kimmel et al. 1993; Edwards et al. 1995). The threshold for producing developmental toxicity appears to be a 2.5°C elevation above the resting, thermoneutral core temperature for short-term or acute exposures (Germain, Webster, and Edwards 1985; Lary et al. 1986). Maintaining colonic temperature by RF radiation exposure at 42°C for 15 minutes or 41°C for 2 hours significantly increased developmental toxicity in rats (Lary et al. 1983).

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Previous research in our laboratories indicates that RF radiation and 2-methoxyethanol (2ME; ethylene glycol monomethyl ether; methyl cellosolve; CAS No. 109-86-4) produce interactive developmental toxicity, including synergism at high exposure levels (Nelson et al. 1991, 1994, 1997a, 1997b). This glycol ether was chosen as a model compound because of its well-characterized teratogenicity in experimental animals (NIOSH 1983, 1991; Nelson et al. 1984, 1989; Hanley et al. 1984; Horton et al. 1985; Hardin and Eisenmann 1987; Sleet, Greene, and Welsch 1987, 1988) and speculated teratogenicity in humans (Bolt and Golka 1990). Adverse reproductive effects have been reported to be associated with potential exposures to 2ME or certain other glycol ethers in the semiconductor industry (Rudolf and Swan 1986; Paustenbach 1988; Pastides et al. 1988; Correa et al. 1996; Gray et al. 1996; Hammond et al. 1996; Lumm, Kutcher, and Morris 1996; Pinney and Lemasters 1996; Schenker 1996; Swan and Forest 1996).

Methanol is also known to be teratogenic at relatively high levels in experimental animals. Nelson et al. (1985) reported that inhalation exposure of Sprague-Dawley rats to 10,000 or 15,000 ppm methanol for 7 h/day throughout gestation produced malformations in the offspring (no defects were seen in the 5000-ppm group). Infurna and Weiss (1986) exposed pregnant rats to methanol in drinking water from gestation days 15 to 17 or 17 to 19. Offspring from both exposure periods showed transient behavioral effects soon after birth, but no persistent effects were observed. Investigating 1000, 2000, 5000, 7500, 10,000, and 15,000 ppm methanol, Rogers et al. (1993) reported a no-observable-adverse-effect level (NOAEL) for developmental toxicity of 1000 ppm methanol for 7 h/day on gestation days 6 to 15 in CD-1 mice. This study also included a small number of mice administered 2 g/kg methanol twice daily (7 hours apart) via oral gavage on days 6 to 15, and this dose was reported to induce developmental toxicity similar to that seen in animals exposed to 10,000 ppm methanol by inhalation. Bolon et al. (1993) reported that neural tube defects predominated when CD-1 mice were exposed on gestation days 7 to 9 to 10,000 or 15,000 ppm methanol for 7 h/day; limb anomalies were induced following exposure on days 9 to 11, and cleft palate and hydronephrosis were observed after exposure during either period. Bolon, Welsch, and Morgan (1994) described the pathogenesis of neural tube defects in mice following inhalation exposure to 15,000 ppm methanol for 6 h/day on gestation days 7 to 9 in CD-1 mice. Dorman et al. (1995) studied the role of formate in methanol-induced exencephaly in CD-1 mice, and concluded that the parent compound, not formate, was responsible for the pathogenesis. Fu et al. (1996) reported that marginal folate deficiency significantly increased the developmental toxicity of methanol in CD-1 mice. Weiss et al. (1996; see also Stern et al. 1996, 1997) exposed pregnant Long-Evans rats to 0 or 4500 ppm methanol for 6 h/day from gestation day 6 through postnatal day 21 (both dams and pups being exposed following parturition). They found that pup blood methanol levels were about twice as high as maternal blood levels. Burbacher et al. (1999) investigated the effects of inhalation exposure of nonhuman primates to methanol. Levels of methanol ranged from 0 to 1800 ppm methanol, administered for 2 h/day, 7 days/week prior to and throughout pregnancy. Offspring were examined for birth defects, and were subjected to neurobehavioral testing in early life (further testing may be completed on these animals). No birth defects were observed. Behavioral assessments detected some differences from control, and a wasting syndrome was observed in female offspring at approximately 1 year of age. Thus, these authors suggest the need for further research on the developmental toxicity of methanol.

The present research was conducted to investigate if the interactive effects noted for RF radiation and 2ME are unique to these agents, or if another solvent would interact with RF radiation or 2ME.

MATERIALS AND METHODS

Our experimental animals, exposure system, and procedures were identical to those described previously (Nelson et al. 1994, 1997a, 1997b, 1999). The experimental animals were treated humanely under a study protocol approved by the National Institute of Occupational Safety and Health (NIOSH) Institutional Animal Care and Use Committee (IACUC). Briefly, virgin female (175–200 g) and breeder male (275–300 g) CD Sprague-Dawley rats (VAF/plus; Charles River Breeding Laboratories, Wilmington, MA) were maintained at 24° ± 2°C and 50% ± 10% humidity. Feed was Ziegler certified laboratory rat chow, with tap water available ad libitum and room lighting was on from 7:00 AM to 7:00 PM. During the 2-week quarantine period, quality control tests were conducted to ensure that rats used in the study were healthy and that specific pathogens were not introduced into the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) certified animal facility. For breeding, females were placed individually with males in the afternoon, and the paper under each male's cage was examined the following morning for vaginal plugs. Vaginal smears were taken from females having no plugs to evaluate the presence of sperm or estrus cycle of the female. Presence of vaginal plugs or sperm marked day 0 of gestation.

Rats were irradiated in either of two matched RF near-field synthesizer facilities operated in the dominant electric field mode under continuous wave conditions at a frequency of 10 MHz. The frequency was controlled accurately (frequency resolution to 1 Hz at 10 MHz) by a Hewlett-Packard Model 8660C or 8660D synthesized signal generator. The signal from the generator was amplified by an Amplifier Research Model 1000L or 200L linear amplifier to provide power to the near-field synthesizer. The near-field synthesizers were enclosed within copper screen wire chambers (Ark Electronics Corp., Model A273 and PSS7S10) to reduce interference from outside RF radiation signals and to shield personnel against RF radiation exposure outside of the system. These copper wire chambers were housed in Forma Scientific Model 7010 or 74668 environmental

chambers. All exposures were conducted at ambient temperatures of 24° ± 1°C and a relative humidity of 50% ± 10%. The air exchange rate in the 33-m^3 environmental chambers was approximately 0.4 m^3/min, with nondetectable air velocity at the location of the animal.

Each rat was irradiated once, without anesthesia, in a cylindrical Plexiglas holder perforated with 12-mm holes. The holders were designed to prevent the rat from changing its orientation relative to the RF field and to allow circulation of air about the rat. Each rat was oriented so that its long axis (length) was parallel to the incident electric field to maximize RF radiation absorption. An RF-nonperturbing temperature probe (Luxtron Corp., model MPM; calibration accuracy of ±0.2°C) covered by a sterile closed-end catheter was inserted 5 cm into the animal's colon, and secured with elastic adhesive tape. The tail was taped under the abdomen, and the animal was placed in a mesh bag to prevent the tail and paws from protruding outside the animal holder. Computer-controlled systems monitored the colonic temperature of the irradiated rats and controlled the RF output power such that the colonic temperature was maintained to within ±0.2°C of the target temperature. The output power of the RF radiation source initially was set to provide an SAR (specific absorption rate) of about 8 W/kg to raise the animal's colonic temperature from its normal baseline of approximately 38°C (or the methanol-induced hypothermia) to 41.0°C (requiring approximately 20 minutes). Once the target colonic temperature was reached, the RF radiation output power was adjusted to maintain the target colonic temperature for 60 minutes, with the SAR varying from 0.8 to 7.9 W/kg. We previously found that time-averaged SARs required to achieve or maintain colonic temperatures at 42.0°C were not affected by 2ME exposure (Nelson et al. 1994). Sham-exposed animals were handled identically to the RF-exposed animals except that there was no RF power input and hence no colonic temperature elevation.

2ME (Fisher Scientific, Fair Lawn, NJ, certified grade) at 100 mg/kg was prepared in distilled water based on administration of 10 ml liquid/kg body weight, and was verified by flame ionization gas chromatography to be within 10% of the target concentration by the NIOSH Division of Physical Sciences and Engineering.

Methanol (Fisher Scientific, HPLC grade; 0, 2, or 3 g/kg) was diluted by one half with distilled water based on administration of 1 ml methanol/kg body weight. Rats were gavaged with the specified dose of methanol immediately prior to preparing the animals for irradiation (except as noted below; preparation time of 5–10 minutes) on gestation day 9 (the most susceptible time of RF radiation teratogenesis, yielding primarily craniofacial malformations; Lary et al. 1982). The specified dose of methanol, along with 2ME, was given in the morning of gestation day 13 (the most susceptible time of 2ME teratogenesis on digit formation). In our pilot study, we observed that 3 g/kg methanol produced approximately a 1° to 1.5°C drop in colonic temperature, reaching the nadir 1 to 2 hours after methanol administration. Because RF radiation is hyperthermic, it was of interest to determine if a different interactive effect would be observed if the rats reached the hypothermic nadir prior to initiating the RF radiation-induced hyperthermia. Hence, groups of rats were exposed at approximately the same time (viz., "close" in time, with the second dose of methanol given 6 hours after the RF or 2ME; the time of 6 hours was selected for practical, experimental reasons—desiring a maximal separation time during the normal 8-hour work day, but with two dose times possible), and other groups were exposed 1.5 hours following the initial dose of methanol ("apart," with the second dose of methanol given 4.5 hours after the RF or 2ME). Although there was no reason to expect this temperature difference to affect the interactions with 2ME, we included similar groups to be consistent between RF radiation and 2ME. For ease of discussing the statistical treatment of the data, the two teratogens we had used in our previous studies are referred to as "prototype" teratogens, and methanol as the "new" teratogen.

Dams were sacrificed on day 20 (as typical in teratology), and fetuses were removed serially. Fetuses were blotted dry, weighed, and examined for external malformations by trained observers who were blind to the treatment conditions of the fetuses at the time of observation. We previously reported that digit malformations were more easily detected in fresh tissue than fixed tissue (Nelson et al. 1994). Hence we did not include examination for skeletal or visceral malformations, nor for functional deficits, in the present study.

A split-plot design was used for the study (Table 1). The whole plot represented the level of separation in time of exposure to the two teratogenic agents (close or apart). The other two independent variables, the "prototype" teratogen and the new teratogen, were presented factorially within each whole plot. The "prototype" teratogen was either RF radiation administered on day 9 (as a sham or maintaining colonic temperature at 41°C for 1 hour), or 2ME administered on day 13 (at levels of 0 or 100 mg/kg). In both cases, the new teratogen (viz., methanol) was administered before the prototype teratogen, either immediately before or 1.5 hours before. Methanol was administered at 0, 2, or 3 g/kg (two doses of 0, 2, or 3 g/kg administered 6 hours apart). Ten blocks of dams (a block consisting of of two whole plots, one "apart" and one "close," ordered randomly) were successively run over a period of about 8 months. All treatment combinations appeared in each block. Dams were randomly assigned to treatment conditions within a block.

Statistical Analyses

Two methods were used to analyze the data: (1) generalized estimating equations (Liang and Zeger 1986) for fetal responses of a quantal nature (malformations, resorptions, and affected [malformed or resorbed]) and (2) mixed models (Laird and Ware 1982) for responses of a continuous nature (weight). For the quantal responses, the data were binary responses, y_{ij}, where $y_{ij} = 1$ if a response (e.g., presence of malformation) occurred in fetus j of litter i and $y_{ij} = 0$ if a response did not occur. In applying generalized estimating equations (GEE), the binomial

TABLE 1
Distribution of animal exposures

	Day 9 exposure			
	Close		Apart (1.5 h delay)	
	RF sham	RF (41°C × 1 h)	RF sham	RF (41°C × 1 h)
Distilled water control	10 (target)	10	10	10
Methanol (2 g/kg 2 × /day)	10	10	10	10
Methanol (3 g/kg 2 × /day)	10	10	10	10
	Day 13 exposure			
	Close		Apart (1.5 h delay)	
	Distilled water	2ME (100 mg/kg)	Distilled water	2ME (100 mg/kg)
Distilled water control	10	10	10	10
Methanol (2 g/kg 2 × /day)	10	10	10	10
Methanol (3 g/kg 2 × /day)	10	10	10	10

distribution and the logit link were used, thus

$$\log_e(\mu_{ij}/(1-\mu_{ij})) = \beta_0 + \beta_1 x_{ij1} + \beta_2 x_{ij2} + \beta_3 x_{ij3} + \beta_4 x_{ij4} + \cdots + \beta_{n_p} X_{ijn_p}$$

where

$\mu_{ij} = E(y_{ij})$ = mean of y_{ij}
β_0 = intercept
β_1 = parameter for effect of the "prototype" teratogen (RF radiation on day 9 and 2ME on day 13)
β_2 = parameter for effect of methanol
β_3 = parameter for interactive effect of "prototype" teratogen (RF radiation or 2ME) and methanol ($x_{ij3} = x_{ij1} * x_{ij2}$)
β_4 = parameter for effect of separation ($x_{ij4} = 1$ if "apart," $x_{ij4} = 0$ if "close"); in the estimation procedure "separation" was a qualitative (class) variable
$\beta_5, \ldots, \beta_{n_p}$ = parameters for effect of whole plot, which is nested within level of separation ($x_{ij5} - x_{ijn_p}$ are dummy variables); for day 9 there were 20 whole plots and for day 13 there were 17 whole plots; in the estimation procedure whole plot was a qualitative (class) variable. These parameters were included in order to model the experimental design correctly. The purpose of using whole pots was to take into account possible unanticipated factors that might have impinged on the results over time
x_{ijp} = level of independent variable corresponding to β_p. The variables x_{ij1} and x_{ij2} were centered. The variable $x_{ij1} = x'_{ij1} - \bar{X}_1$, where $x'_{ij1} = 1$ if the "prototype" teratogen (RF radiation or 2ME) was present, and $x'_{ij1} = 0$ if the "prototype" teratogen was absent. Similarly, $x_{ij2} = x'_{ij2} - \bar{X}_2$, where x'_{ij2} = level of methanol (0, 2, or 3 g/kg)

For GEE the working correlation matrix was assumed to be exchangeable. Calculations were performed using PROC GENMOD in SAS (SAS Institute Inc., Cary, NC). The main goal was to test for an interactive effect between the prototype teratogen and methanol. Tests for interactive effects, as well as other effects, were conducted using Wald tests on parameter estimates. The effect of whole plot was assessed using a generalized score test (Rotnitzky and Jewell 1990). Results are presented for day 9 for resorptions and for affected fetuses; day 13 results are presented for resorptions, malformations, and affected fetuses. The model for malformations on day 9 did not converge, presumably because of the relatively low number of malformations on day 9. (Because malformations are observed only in "live fetuses," [not resorbed fetuses] and resorptions are based on the total number of conceptuses, "affected fetuses" are not a simple numerical summary of the fraction resorbed plus the fraction malformed.)

In analyzing fetal weights, all of the factors were assumed to be fixed except for whole plot. The correlation among fetuses in the same litter was assumed to be exchangeable. The REML method of estimation was used. The mixed model was:

$$y_{ijklmn} = \mu + \alpha_k + \beta_l + \gamma_{kl} + \eta_m + W_{n(m)} + e_{ijklmn}$$

where

y_{ijklmn} = weight of fetus j in litter i for treatment combination k, l, m, n as defined below
μ = overall mean
α_k = fixed effect due to either RF radiation (day 9) or 2ME (day 13); $k = 1, 2$
β_l = fixed effect due to methanol; $l = 1, 2, 3$
γ_{kl} = fixed effect due to interaction of prototype teratogen (RF radiation or 2ME) and methanol
η_m = fixed effect due to separation (close or apart); $m = 1, 2$

$W_{n(m)}$ = random effect due to whole plot (nested within separation). This effect was included in order to model the experimental design correctly. The purpose of using whole plots was to take into account possible unanticipated factors that might have impinged on the results over time

e_{ijklmn} = random error term

Calculations were performed in PROC MIXED in SAS.

RESULTS

Approximately 95% of the dams mated in this study were pregnant at terminal sacrifice. The RF radiation approached a maternally lethal level. In order of increasing dose of methanol, 1, 2, and 2 rats died when RF was given immediately following chemical dosing. Delaying the RF for 1.5 hours following methanol increased maternal lethality to 1, 5, and 8 rats (dams that died were replaced, such that the resulting *N*s were 10/group). Two deaths occurred in rats given methanol and 2ME (1 each in the groups given 2 and 3 g/kg methanol followed closely by 2ME—likely not treatment related). There were no other indications of maternal toxicity.

The mean initial body temperature of rats given methanol 1.5 hour prior to RF radiation was 37.4°C. This is nearly a degree lower than that seen with those not given methanol (38.3°C). Nonetheless, the SAR to reach and maintain 41.0°C was not significantly affected by methanol exposure.

Day 9 Exposures (RF Radiation + Methanol)

The results for day 9 are shown in Figure 1, and significant effects are presented in the tables below. The results for resorbed fetuses are shown in Table 2. Hence, the presence of RF radiation, administration of methanol, or the two teratogens being administered apart in time significantly increased the rate of resorption. (A positive estimate for a parameter means that there will be an increase in $\hat{\mu}_{ij}$.) According to the generalized score test, the whole plot effect for resorbed fetuses on day 9 was not significant ($\chi^2 = 17.83$, $df = 18$, $p = .4672$; this suggested that there was no drift in results over the course of the experiment). The actual fractions of fetuses that were resorbed for day 9 for different levels of the significant effects are presented in Table 3.

The results for affected fetuses on day 9 are presented in Table 4. The pattern shown in Table 4 is similar to that shown for

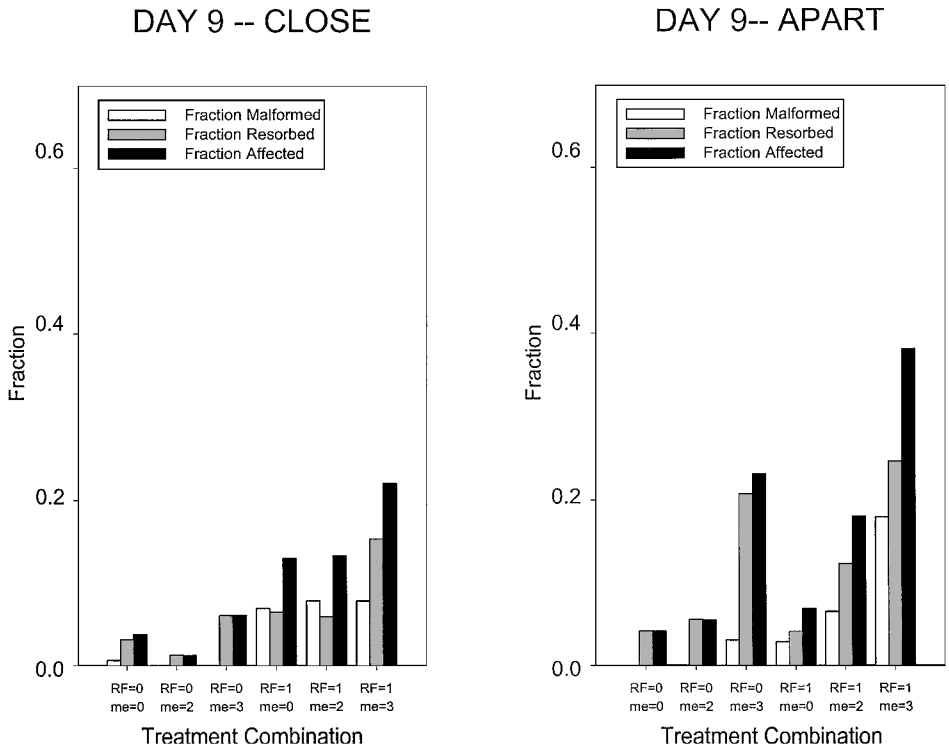

FIGURE 1

Summary of data after exposure of rats on gestation day 9 to RF radiation (RF0 = sham exposure for 60 minutes; RF1 = maintaining colonic temperature at 41°C for 60 minutes) and/or methanol (me0, 2, and 3 corresponds with administration of 0 [distilled water], 2, and 3 g/kg methanol twice at 6-hour intervals) either with concurrent exposures or delayed by 1.5 hours (see text for details). As presented in the legend, data include fraction of conceptuses resorbed, fraction of fetuses malformed, and fraction of conceptuses affected (conceptually, a summary of the previous two, although resorptions are based on the total conceptuses whereas malformations are based on live fetuses only; hence it is not a simple numerical summary).

TABLE 2
Effects of RF radiation, methanol, their interaction, and separation of exposures on resorptions when maternal rats were exposed on day 9

Effect	Parameter	Parameter estimate	Empirical standard error	95% CI	Z	p value for Wald test
Intercept	β_0	−3.782	0.972	−5.688, −1.876	−3.89	.0001
RF radiation	β_1	0.697	0.284	0.141, 1.252	2.46	.0140
Methanol	β_2	0.564	0.130	0.309, 0.818	4.33	<.0001
RF radiation × Methanol	β_3	−0.020	0.255	−0.520, 0.481	−0.08	.9379
Separation	β_4	2.135	1.043	0.091, 4.180	2.05	.0407

TABLE 3
Fractions of fetuses resorbed following exposure on day 9 for factors that had significant effects

RF level	Methanol level (g/kg)	Separation of exposures	Actual fraction of fetuses resorbed	95% CI for actual fraction
Absent	All	Both	0.070	0.054, 0.086
Present	All	Both	0.112	0.090, 0.133
Both	0	Both	0.045	0.028, 0.062
Both	2	Both	0.059	0.040, 0.078
Both	3	Both	0.162	0.133, 0.192
Both	All	Apart	0.117	0.096, 0.139
Both	All	Close	0.063	0.048, 0.079

TABLE 4
Effects of RF radiation, methanol, their interaction, and separation of exposures on affected fetuses from maternal rats that were exposed on day 9

Effect	Parameter	Parameter estimate	Empirical standard error	95% CI	Z	p value for Wald test
Intercept	β_0	−3.584	0.897	−5.342, −1.825	−3.99	<.0001
RF radiation	β_1	1.323	0.286	0.762, 1.885	4.62	<.0001
Methanol	β_2	0.543	0.130	0.289, 0.797	4.19	<.0001
RF radiation × Methanol	β_3	−0.090	0.253	−0.586, 0.406	−0.35	.7227
Separation	β_4	2.322	1.033	0.297, 4.346	2.25	.0246

TABLE 5
Actual fractions of affected fetuses from maternal rats exposed on day 9 for the independent variables that had significant effects

RF level	Methanol level (g/kg)	Separation of exposures	Actual fraction of fetuses affected	95% CI for actual fraction
Absent	All	Both	0.075	0.058, 0.092
Present	All	Both	0.181	0.155, 0.208
Both	0	Both	0.070	0.050, 0.091
Both	2	Both	0.089	0.065, 0.112
Both	3	Both	0.214	0.181, 0.246
Both	All	Apart	0.154	0.130, 0.178
Both	All	Close	0.097	0.078, 0.116

TABLE 6
Effects of RF radiation, methanol, their interaction, and separation of exposure on fetal weights for rats exposed on day 9

Effect	Numerator *df*	Denominator *df*	*F* value	*p* value
RF radiation	1	1594	0.75	.3872
Methanol	2	1594	19.27	<.0001
RF radiation × Methanol	2	1594	1.46	.2316
Separation	1	18	0.91	.3533

resorbed fetuses, with RF radiation, methanol, and "apartness" associated with increased rates of being affected. The effect of whole plot on affected fetuses on day 9 was not significant ($\chi^2 = 16.08$, $df = 18$, $p = .5869$). Significant effects for the actual fractions of total conceptuses affected following day 9 exposures are shown in Table 5.

The results for fetal weights are shown in Table 6. Thus, the only factor having a significant effect on fetal weights for day 9 exposures was methanol exposure. The actual mean weights and adjusted means obtained from the model for different levels of methanol are shown in Table 7.

Day 13 Exposures (2ME + Methanol)

The results for exposures on day 13 are shown in Figure 2. Again, the results of significant effects are shown in the tables. The results for malformed fetuses from maternal rats exposed on day 13 are shown in Table 8. Thus, there were significant effects due to 2ME, methanol, and their interaction. The positive estimates of the parameters imply increased rates of malformations due to these factors. For the fetuses on day 13, separation time did not have a significant effect on rate of malformations. The whole plot effect for day 13 malformed fetuses was not significant ($\chi^2 = 20.52$, $df = 15$, $p = .1530$). The actual

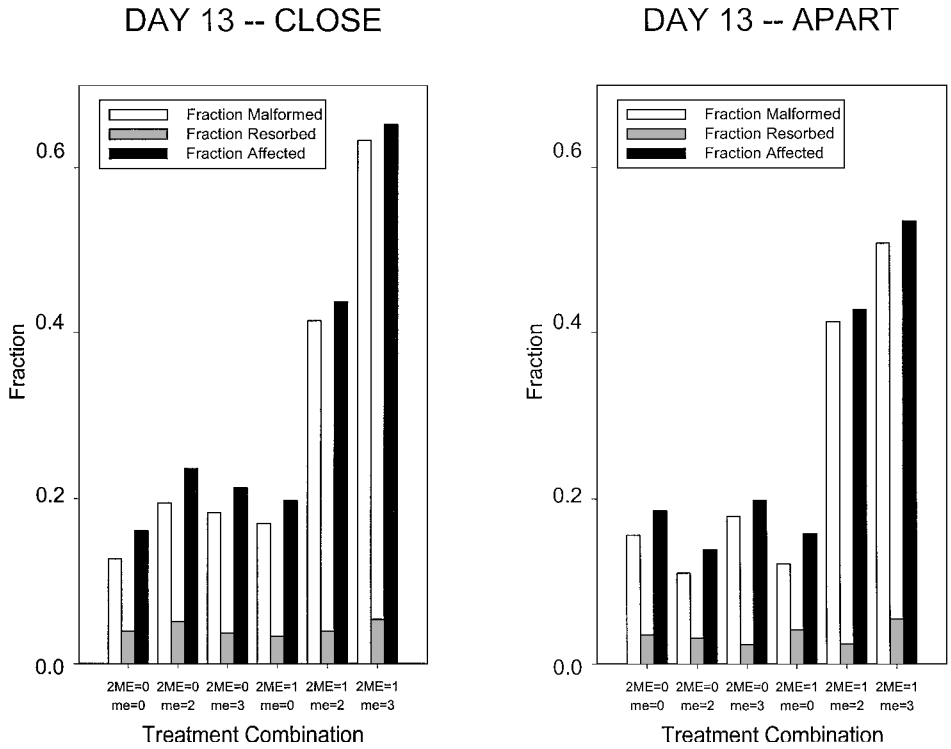

FIGURE 2
Summary of data after exposure of rats on gestation day 13 to 2-methoxyethanol (2ME0 = distilled water; 2ME1 = 100 mg/kg) and/or methanol (me0, 2, and 3 corresponds with administration of 0 [distilled water], 2, and 3 g/kg methanol twice at 6-hour intervals) either with concurrent exposures or delayed by 1.5 hours (see text for details). As presented in the legend, data include fraction of conceptuses resorbed, fraction of fetuses malformed, and fraction of conceptuses affected (conceptually, a summary of the previous two, although resorptions are based on the total conceptuses whereas malformations are based on live fetuses only; hence it is not a simple numerical summary).

TABLE 7
Means of fetal weights for different levels of methanol for fetuses exposed on day 9

Methanol level (g/kg)	Actual mean weight of fetuses (g)	95% CI for actual means	Adjusted mean of fetal weight (from mixed model)	95% CI for adjusted mean
0	3.661	3.632, 3.689	3.663	3.571, 3.755
2	3.551	3.522, 3.580	3.523	3.428, 3.618
3	3.253	3.218, 3.287	3.256	3.162, 3.349

TABLE 8
Effects of 2ME, methanol, their interaction, and separation of exposures on malformations in fetuses obtained from maternal rats exposed on day 13

Effect	Parameter	Parameter estimate	Empirical standard error	95% CI	Z	p value for Wald test
Intercept	β_0	−1.676	0.475	−2.608, −0.745	−3.53	.0004
2-ME	β_1	1.168	0.168	0.839, 1.498	6.95	<.0001
Methanol	β_2	0.424	0.068	0.290, 0.559	6.19	<.0001
2-ME × Methanol	β_3	0.573	0.136	0.307, 0.840	4.21	<.0001
Separation	β_4	0.389	0.662	−0.909, 1.686	0.59	.5571

fractions of malformed fetuses for day 13 exposures are shown in Table 9.

For day 13 the results for resorptions are presented in Table 10. The whole plot effect for day 13 resorbed fetuses was not significant ($\chi^2 = 11.32$, $df = 15$, $p = .7298$). Thus, none of the factors had a significant effect on resorptions from day 13 exposures. We omit the actual fractions for day 13 resorptions because of the lack of significant effects.

TABLE 9
Actual fractions of malformed fetuses from maternal rats exposed on day 13 for the independent variables that had significant effects

2-ME	Methanol level (g/kg)	Actual fraction of all fetuses malformed	95% CI for actual fraction
Absent	All	0.161	0.139, 0.182
Present	All	0.389	0.360, 0.418
Both	0	0.145	0.119, 0.171
Both	2	0.289	0.258, 0.321
Both	3	0.379	0.344, 0.414
Absent	0	0.136	0.100, 0.172
Absent	2	0.162	0.126, 0.199
Absent	3	0.182	0.143, 0.221
Present	0	0.154	0.116, 0.192
Present	2	0.413	0.365, 0.462
Present	3	0.586	0.535, 0.636

For fetuses exposed on day 13, the significance of different factors with regard to being affected is shown in Table 11. The tests showed highly significant effects due to 2ME, methanol, and their interaction. No significant effect was shown for separation. The whole plot effect for affected fetuses from maternal rats exposed on day 13 yielded a p value of .0601 ($\chi^2 = 24.30$, $df = 15$). The actual fractions of affected fetuses for day 13 are shown in Table 12.

Fetal weights for day 13 exposures are shown in Table 13. Thus, we see highly significant effects on fetal weight due to each of the teratogens acting alone, but none due to interaction or separation. The actual mean weights for the day 13 fetuses and adjusted means obtained from the model are presented in Table 14.

DISCUSSION

Overall we see that the patterns of effects after exposure on gestation days 9 and 13 were rather different—probably due to the effects of the teratogens acting at different developmental stages. For example, the primary impact of exposures on day 9 was on resorptions, whereas on day 13 the main impact was on malformations. Another difference was that separation in time of the exposures had a significant effect on day 9, but not on day 13. A third difference was the presence of a significant positive interactive effect on day 13 between 2ME and methanol, but not on day 9 between RF radiation and methanol. Finally, RF radiation did not significantly affect fetal weight (in contrast to some of our previous studies), but 2ME did. Ideally, we would have administered the teratogens on the same day of gestation, but the

TABLE 10
Effects of 2ME, methanol, their interaction, and separation of exposures on resorptions in maternal rats exposed on day 13

Effect	Parameter	Parameter estimate	Empirical standard error	95% CI	Z	p value for Wald test
Intercept	β_0	−3.836	0.456	−4.730, −2.942	−8.41	<.0001
2ME	β_1	0.060	0.214	−0.360, 0.480	0.28	.7802
Methanol	β_2	0.059	0.088	−0.113, 0.231	0.67	.5000
2ME × Methanol	β_3	0.157	0.174	−0.184, 0.498	0.90	.3679
Separation	β_4	0.141	0.689	−1.209, 1.492	0.21	.8374

TABLE 11
Effects of 2ME, methanol, their interaction, and separation of exposures on affected fetuses from maternal rats exposed on day 13

Effect	Parameter	Parameter estimate	Empirical standard error	95% CI	Z	p value for Wald test
Intercept	β_0	−1.527	0.453	−2.416, −0.639	−3.37	.0008
2ME	β_1	1.051	0.149	0.758, 1.344	7.03	<.0001
Methanol	β_2	0.383	0.060	0.265, 0.501	6.36	<.0001
2ME × Methanol	β_3	0.557	0.120	0.322, 0.792	4.64	<.0001
Separation	β_4	0.369	0.634	−0.872, 1.611	0.58	.5600

simplest way to detect interactions is to administer the agents when at least one of the teratogens exerts its maximal effect (Nelson 1994). We used resorptions, external malformations, and fetal weights as our measures of developmental toxicity. Previous research had demonstrated that RF radiation produced its maximal effect on external malformations (viz., craniofacial malformations) when administered on day 9, and that 2ME exerted its maximal effect (viz., digit malformations) when administered on day 13.

Some of these patterns could be predicted, but others would not. This complicates predicting when to expect interactions amongst various agents. For example, the lack of interaction between methanol and RF radiation, but significant interaction with methanol and 2ME is different from our previous research investigating interactions of acetylsalicylic acid with RF radiation on day 9 and interactions with 2ME on day 13 (Nelson, Snyder, and Shaw 1999). One might logically predict that RF radiation, a hyperthermic agent under certain conditions (which may not be seen to a significant degree with occupational exposures), would interact with agents which produce hypo- or hyperthermia, but the small degree of hypothermia produced by the doses of methanol we used did not yield significant interactions. This lack of effect, however, should not be construed to suggest that temperature effects may not play a significant role in interactions. The literature is replete with studies which implicate altered body temperature as a primary interacting agent with toxic (e.g., Keplinger, Lanier, and Deichmann 1959; Weihe 1973) and teratogenic agents (e.g., Ferm and Kilham 1977; Ferm and Ferm 1979; Hanlon and Ferm 1986; Shiota et al. 1988; Nelson 1994). It should also be noted that (apparently) nonthermic levels of

TABLE 12
Actual fractions of affected fetuses from maternal rats exposed on day 13 for the independent variables that had significant effects

2ME	Methanol level (g/kg)	Actual fraction of fetuses affected	95% CI for actual fraction
Absent	All	0.193	0.170, 0.215
Present	All	0.414	0.386, 0.442
Both	0	0.177	0.149, 0.204
Both	2	0.316	0.285, 0.348
Both	3	0.406	0.371, 0.440
Absent	0	0.169	0.130, 0.207
Absent	2	0.199	0.160, 0.237
Absent	3	0.209	0.168, 0.249
Present	0	0.185	0.144, 0.225
Present	2	0.433	0.385, 0.481
Present	3	0.608	0.559, 0.657

TABLE 13
Effects of 2ME, methanol, their interaction, and separation of exposure on fetal weights for rats exposed on day 13

Effect	Numerator *df*	Denominator *df*	*F* value	*p* value
2ME	1	2231	75.62	<.0001
Methanol	2	2231	59.29	<.0001
2ME × Methanol	2	2231	0.44	.6448
Separation	1	15	0.46	.5081

TABLE 14
Means of fetal weights for different levels of 2ME and methanol for fetuses exposed on day 13

2ME	Methanol level (g/kg)	Actual mean weight of all fetuses (g)	95% CI for actual means	Adjusted mean of fetal weight (from mixed model)	95% CI for adjusted mean
Absent		3.395	3.373, 3.418	3.390	3.318, 3.462
Present		3.073	3.051, 3.096	3.064	2.992, 3.135
	0	3.497	3.472, 3.523	3.494	3.411, 3.576
	2	3.216	3.189, 3.244	3.199	3.119, 3.278
	3	3.010	2.985, 3.036	2.988	2.907, 3.068

magnetic radiation from video display terminals reportedly enhanced the developmental toxicity of cytosine arabinoside in mice (Chiang et al. 1995; cf., Marcickiewicz et al. 1986). Other investigators reported that nonthermic RF radiation produced adverse effects in chick embryos (Saito, Suzuki, and Motoyoshi 1991; Saito and Suzuki 1995). However, these apparent nonthermal effects are not consistent with the majority of research in RF radiation teratology, so more research is needed.

Hyperthermia may be induced by various means in addition to RF radiation. For example, administration of amphetamine or methamphetamine to experimental animals can produce hyperthermia (Bowyer et al. 1992; 1994; Bowyer and Holson 1995; Fukumura et al. 1998). Pesticides such as chlorpyrifos can induce hyperthermia, although this may be somewhat of a "rebound effect" after the initial hypothermia (Gordon 1994; Rowsey and Gordon 1997). Fever is known to change pharmacokinetics and pharmacodynamics of some drugs (Mackowiak 1991). Stress-induced hyperthermia can be elicited by individually removing rodents from the group home cage (Van Der Heyden, Zethof, and Olivier 1997).

Hypothermia has not been reported as frequently in the toxicology literature. We had not noted reports of methanol-induced hypothermia, but such effects could be expected based on the hypothermic effects of structurally related alcohols (e.g., Wimer, Russell, and Kaplan 1983). The relative timing of exposures and where the dosings fit on hypo/hyperthermic curves, along with different times between dosings, would be expected to affect potential interactions. As noted earlier in the paper, methanol produced nearly a 1°C temperature decrease. Strictly on thermodynamic principles, one would predict that the average SAR to reach and maintain a colonic temperature of 41.0°C would be inversely proportional to the initial body temperature. Because we did not see statistically significant differences in SAR among treatment groups, we assume that the variability associated with the group SARs masked potential small differences in SAR. Whether or not nondetectable differences in absorbed RF energy accounted for our observed differences from separation time remains to be determined. The cause-effect relationships and interactions of hyper- and hypothermia present a potentially fruitful area of further research in toxicology in general, and particularly in developmental toxicology. Certain worker populations can be exposed to extreme environmental temperature conditions, especially on a seasonal basis, and how these extreme conditions may interact with the toxicity of other agents on these workers is largely unstudied.

Thus, we conclude that there was no interaction of RF radiation and methanol under conditions of the present study, in spite of the fact that both agents exerted an effect individually. In contrast, significant interactions were observed between 2ME and methanol. Whether or not these results in rats have direct implications for risk assessment of the agents studied remains to be determined. For our purposes, it is sufficient to recommend that additional research be undertaken to investigate interactions among occupational agents, and that potential interactions be considered when developing both physical agent and chemical agent exposure standards and intervention strategies.

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