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Interactive Developmental Toxicity of Radiofrequency Radiation and 2-Methoxyethanol in Rats[1]

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ABSTRACT Concurrent exposures to chemical and physical agents occur in the workplace; exposed workers include those involved with the microelectronics industry, plastic sealers, and electrosurgical units. 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 radiofrequency (RF; 10 MHz) radiation, which also induces hyperthermia and is teratogenic to exposed animals, and the industrial solvent, 2-methoxyethanol (2ME), produces enhanced teratogenicity in rats. The present study replicates and extends the previous research investigating the enhanced teratogenicity of combined RF radiation and 2ME exposures. The interactive dose-related teratogenicity of RF radiation (sham exposure or maintaining colonic temperatures at 42.0°C for 0, 10, 20, or 30 min) and 2ME (0, 75, 100, 125, or 150 mg/kg) was investigated by administering various combinations of RF radiation and 2ME to groups of rats on gestation days 9 or 13; gestation-day 20 fetuses were examined for external, skeletal, and visceral malformations. The results are consistent with and extend our previous research findings. Synergism was observed between RF radiation and 2ME for some treatment combinations, but not for others. The study also clarified which gestational periods, RF radiation exposure durations, and 2ME doses would be most informative in future interaction studies to determine the lowest interactive effect level. Day 9 exposures generally evidenced little effect by 2ME, either by itself or in combination with RF radiation. In contrast, day 13 exposures resulted in highly significant effects from 2ME and RF radiation. The structures showing strong evidence of effects from both 2ME and RF radiation after exposure on gestation day 13 were the forepaw digits, forepaw phalanges, hindpaw digits, hindpaw phalanges, hind limbs, metacarpals, and metatarsals. Statistical analyses did not show a global synergistic effect, but did show evidence for a synergistic effect at intermediate levels of the dose ranges. Future research will address potential interactions at lower doses.

Radiofrequency (RF) radiation, defined as the portion of the electromagnetic spectrum in the frequency range from 3 kHz to 300 MHz, is used frequently in the workplace (NIOSH/OSHA, '79; Joyner and Bangay, '86; Conover et al., '86, '92). An estimated 3.4 million workers are exposed occupationally to low frequency RF radiation emitted by RF dielectric heaters and sealers, induction heaters, diathermy and electrosurgical equipment, and a variety of other devices (Centaur, '82; Stewart and Elkington, '85). Many of these workers are exposed to electric and/or magnetic field strengths in excess of current exposure standards (Ruggera, '77; Conover et al., '81, '92; Cox et al., '81, '82; Centaur, '82).

Several reports suggest that RF radiation may be teratogenic in humans (e.g., Marchese, '53; Cocozza et al., '60). Other reports suggest that RF radiation may produce spontaneous abortions in women given diathermy treatments just prior to, or during, early pregnancy (Rubin and Erdman, '59; Imrie, '71). Hoffman and Dietzel ('66) reviewed the physiological consequences of shortwave heating of the uterus and concluded that shortwave diathermy is contraindicated during pregnancy because of the adverse effects on the conceptus.

Lary et al. ('82) described the teratogenicity of RF radiation in rats. In that experiment, rats were exposed to RF radiation at 27 MHz with a dominant magnetic field strength of 55 amps/meter (A/m) for approxi-

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mately 30 min on gestation days 1, 3, 5, 7, 9, 11, 13, or 15. Exposures were terminated when the maternal colonic temperature reached 43°C (which approaches the maternally lethal level). The incidence of external, visceral, and skeletal malformations was increased in exposed litters, particularly from exposure on days 9, 11, and 13. The types of malformations were dependent on the day of radiation administration, with exposure on day 9 producing the highest incidence and largest variety of malformations (most frequent were microphthalmia, anophthalmia, exencephaly, protruding tongue, and skeletal malformations). In a subsequent study of rats exposed to RF radiation on gestation day 9, Lary et al. ('86) found that when exposures were terminated at a colonic temperature of 43°C, 60% of the fetuses were malformed. When the exposures were terminated at a colonic temperature of 42°C, the percentage of malformed fetuses was only about 8%. If terminated when the colonic temperature reached 41°C, there was no increase in malformations (i.e., about 1% of the fetuses were malformed, which was the same level seen in controls).

Increasing the duration of exposure of rats to RF radiation increases the incidence of malformations (Lary et al., '83). For example, maintaining the core temperature at 42°C for 15 min (versus terminating exposure when the temperature just reached 42°C) increased the incidence of external malformations from 5 to 53%. Maintaining the core temperature at 41°C for 2 hr (versus terminating exposure when the temperature just reached 41°C) also significantly increased the incidence of skeletal malformations (from 2 to 6%), but the increase was less dramatic for external malformations (increased from about 1 to 2%) and visceral malformations (no change from 2%). The Lary et al. studies cited above ('83, '86) were conducted at 27 MHz with dominant magnetic field exposures. Similar trends were noted with dominant electric field exposures at 27 MHz (Brown-Woodman et al., '88) and at 10 MHz (Nelson et al., '91b). Thus, it appears that the developmental toxicity of RF radiation is associated with the degree and duration of hyperthermia (Lary and Conover, '87).

Other studies (Lacy, '81) demonstrated that raising the core temperature to 43°C, either by RF radiation or by hot water bath immersion, produced malformations in 64% of the conceptuses. Administration of an anesthetic (which produced hypothermia) followed by RF radiation exposure (which elevated the temperature to normal) produced no increase in embryotoxicity above control levels. These data also indicate that RF radiation-induced teratogenic effects are related to the degree of hyperthermia, in contrast to nonthermic properties of the radiation.

Hyperthermia produces congenital malformations in experimental animals, and it has been hypothesized to produce malformations in humans (Edwards, '86; Lary, '86; Warkany, '86; Kimmel et al., '93). The teratogenic effects in animals seen after RF radiation-induced hyperthermia are strikingly similar to those observed by Germain et al. ('85) using hot water bath immersion. The latter investigators noted that the 2.5°C elevation of core temperature (effect threshold, from 38.5 to 41.0°C; the same threshold as reported by Lary et al., '86) also can be produced in humans under a variety of conditions (Germain et al., '85). These conditions include fever (frequently a 3°C rise in core temperature), marathon running (core temperature elevations as high as 4.6°C), and misuse of saunas and hot tubs (core temperature elevations of 3.2°C). In addition, Brown-Woodman et al. ('88) cited calculations by Joyner ('88) which predicted that occupational exposures to RF radiation in plastic sealer operators could result in core temperature elevations in excess of 2.5°C in a single working shift. The figure of 2.5°C may be erroneous because the researchers incorrectly assumed that the RF energy absorption rate is uniform within the operator. Some data are available for measured surface temperature increases in the wrists and ankles of human volunteers after RF radiation exposure (Gandhi et al., '86; Chen and Gandhi, '88; '89; Conover et al., '92), but core temperature elevation data are needed for RF plastic sealer operators and other workers exposed to RF radiation before the occupational health implications of the present study results can be completely determined.

In addition to RF radiation exposures, many workers are exposed concurrently to industrial chemicals. An example is the electronics industry, where a high percentage of workers are female (Rudolph and Swan, '86). Ladou ('83) estimated that over 500,000 persons employed in the microelectronics industry are potentially exposed to various industrial chemicals; about 55,000 of these employees work directly in producing semiconductor chips, with potential exposures to both industrial chemicals and RF radiation. Extensive OSHA surveys (e.g., OSHA, '83) document that high RF radiation exposures (at or near occupational exposure standards) occur in the semiconductor industry during diffusion, sputtering, and related operations (Ungers and Jones, '86; Pastides et al., '88). Pastides et al. ('88) reported that female workers involved in the diffusion and photolithographic processes used in manufacturing semiconductor chips had an elevated risk of spontaneous abortion. A final report to the Semiconductor Industry Association (Schenker, '92) reported evidence for an increase in spontaneous abortions among women who work in silicon wafer manufacturing fabrication rooms compared to women in the industry who work in nonfabrication locations. In addition to potential exposure to RF radiation (Cohen, '86), these women are exposed to glycol ethers, isopropanol, xylene, and toluene—all of which have been reported to be teratogenic in animals (Cone, '86). A study involving an even larger population of semiconductor manufacturing workers found an increased risk of spontaneous abortion among

women who used glycol ether solvents versus those who used other solvents (Gray and Corn, '93). Paustenbach ('88) reviewed the developmental risks associated with occupational exposures to glycol ethers in the semiconductor industry. Based primarily on research in experimental animals, he indicated that the short-chain glycol ethers may be of greatest concern.

A second industry for which surveys have reported simultaneous exposure to RF radiation and putative chemical teratogens is the dielectric heating industry, employing about 500,000 workers. Glycol ethers, toluene, xylene, and other chemicals are frequently found in dielectric heating areas in which RF radiation is used to weld or seal vinyl materials (Cox and Conover, '81).

A third occupational setting in which combined RF radiation and chemical exposures occur is operating rooms where electrosurgical units (ESUs) are used extensively to cut and coagulate tissues (in about one-half of all surgeries). High RF radiation exposures have been documented in these units (Fox et al., '76; Ruggera, '77). Several anesthetic gases also found in operating rooms, including nitrous oxide and halothane, are reported to be teratogenic when administered at higher concentrations by inhalation to animals, and are possible human teratogens (Schardein, '93). Isopropyl alcohol, widely used as an antibacterial agent in cleaning operating rooms, is teratogenic when administered at high concentrations by inhalation to animals (Nelson et al., '88). Elevated rates of congenital malformations and spontaneous abortions have been reported for female operating room personnel or wives of male anesthetists (e.g., ASA, '74; Goldstein et al., '76).

Despite recognition that elevated temperatures can be teratogenic and can affect adult toxicity (Keplinger et al., '59; Wiehe, '73), and that concurrent exposures occur frequently in occupational environments, there has been limited investigation of the effect of temperature on the teratogenicity of chemicals (Fraser, '77; Nelson, '94). Ferm and Kilham ('77) observed that hyperthermia acted synergistically with sodium arsenate to increase the teratogenicity in hamsters.[2] Hanlon and Ferm ('86) extended this work, finding that maternal blood arsenate levels were substantially higher (approximately twice as high) for nearly 1 hr following combined treatment than after arsenate exposure alone. Elevated blood levels of the putative teratogen following the combined treatments provide a rationale for the increase in malformations observed. Ferm and Ferm ('79) noted similarly enhanced teratogenic effects for vitamin A and hyperthermia. Shiota et al. ('88) reported that hyperthermia administered concurrently with ethanol to mice produced synergistic teratogenic effects.

[2]Defined by Klaassen and Eaton ('91) and Nelson ('94) as an effect greater than additivity.

We previously reported (Nelson et al., '91a) an enhanced incidence of teratogenic effects when rats were exposed to RF radiation (maintaining colonic temperature at 42.0°C for 30 min) along with the industrial solvent 2-methoxyethanol (2ME; 150 mg/kg by gavage). This glycol ether was chosen as a model compound because of its well-characterized teratogenicity in experimental animals (NIOSH, '83, '91; Nelson et al., '84, '89; Hanley et al., '84; Horton et al., '85; Toraason et al., '85, '86; Hardin and Eisenmann, '87; Sleet et al., '87, '88). Additionally, Bolt and Golka ('90) reported cases involving two human infants whose congenital malformations were postulated to result from maternal exposure to the acetate form of 2ME (which is converted rapidly to 2ME in vivo; Sleet et al., '88). Furthermore, as discussed above, adverse reproductive effects have been reported after potential exposures to this or other glycol ethers in the semiconductor industry. We also anticipated that the results from this model compound (viz., 2ME) would be of relevance to other industrial chemical teratogens.

We found that the incidence of fetal malformations was significantly greater after the combination exposure than after 2ME or RF radiation exposure alone (i.e., 76% of the fetuses were malformed under combined exposure conditions compared to 14% of the fetuses malformed after 2ME administration alone and 30% of the fetuses malformed after RF radiation exposure alone). Additionally, the severity of malformations was also enhanced by the combination exposure (Nelson et al., '91a). Based on the pattern of malformations observed in this study, Nelson et al. ('91a) hypothesized that 2ME administration enhanced the teratogenicity of RF radiation exposure.

The purpose of the present research was to characterize the extent of synergism between these two agents in rats and, ultimately, to estimate the likelihood that the interactive effects of RF radiation and 2ME would be observed in humans at occupationally relevant exposures. Since human data are not available either for the extent of possible core temperature increases produced by occupational exposure to RF radiation or for the blood levels resulting from occupational exposure to 2ME, the present project (including additional ongoing research which will be published separately) focused on determining the lowest effect level of teratogenicity for combined RF radiation and 2ME exposures. A determination of this lowest effect level facilitates a more accurate risk assessment of human exposures to these two agents. In addition, whereas the previous study examined only external malformations, the present study included examination of fetuses for skeletal and visceral abnormalities as well as external malformations. Based on the results in the exploratory study, we hypothesized that the enhanced teratogenicity of combined RF radiation/2ME exposure would depend more on the exposure regimen of RF radiation than on the dose of 2ME. Results of an

investigation to determine the effects of RF radiation on the metabolism of 2ME will be published separately.

MATERIALS AND METHODS

Experimental animals

Virgin female and breeder male CD Sprague–Dawley rats (VAF/plus, 175–200g; 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 lib, and room lighting from 7:00 A.M. to 7:00 P.M. During the 2-week quarantine period, quality control tests were conducted on rats to ensure that specific pathogens were not introduced into the 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 vaginal plugs to evaluate the presence of sperm or estrus cycle of the female. Presence of vaginal plugs or sperm marked day 0 of gestation.

RF radiation treatment conditions

Rats were irradiated in one of two RF near-field synthesizer facilities designed for NIOSH by the National Institute for Standards and Technology (NIST; formerly, the National Bureau of Standards; Greene, '76). The synthesizers were operated in the dominant electric field mode under continuous wave conditions at a frequency of 10 MHz, as in the previous study. The 10-MHz frequency was chosen because it is an intermediate frequency within the range of frequencies at which the common sources of occupational exposure to low frequency RF radiation generally operate (frequencies ranging from 0.1 to 100 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 linear amplifier to provide power to the near-field synthesizer. The electric field strength in volts/meter (V/m) was measured with a Holaday Instruments, Inc. Model 30003 field survey monitor. The magnetic field strength in A/m was measured by a Model MFM-10 magnetic field-strength monitor developed for NIOSH by NIST (Conover et al., '86). 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, including sham exposures of control animals, were conducted at an ambient temperature of 24(±1.0)°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. Sham exposures were conducted outside the actual near-field synthesizer facilities, but inside the environmental chambers for the same duration as the RF-exposed animals. Thus, lighting, ventilation, temperature, humidity, and handling procedures were the same for sham exposures as for RF radiation exposures, but no RF field was present during the sham exposure.

Each rat was irradiated once, without anesthesia, in a cylindrical Plexiglas holder perforated with 12-mm holes. RF energy at 10 MHz passes through the Plexiglas with negligible absorption (Lary et al., '82). 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 so that maximum RF-induced heating was obtained. An RF-insensitive temperature probe (Luxtron Corp., model MPM) covered by a sterile closed-end catheter was inserted 5 cm into the animal's colon, and secured with elastic adhesive tape.

Maintaining colonic temperatures at the desired level was a critical factor in these exposures. Consequently, two essentially identical computer-controlled systems were developed to monitor the colonic temperature of the irradiated rats and control the RF output power such that the colonic temperature was maintained to within ±0.2°C of the target temperature. Two Hewlett Packard 437B power meters, a Compaq Deskpro 386/20 computer, associated software, and a Luxtron Model 750 or 755 RF-insensitive temperature monitor were used to control the power output from the HP 8660C or 8660D synthesized signal generator. The output power of the RF radiation source initially was set to provide a specific absorption rate (SAR) of 6.6 W/kg to raise the animal's colonic temperature from its normal baseline of approximately 38–42.0°C (requiring approximately 30 min). Once this colonic temperature was reached, the RF radiation output power was adjusted to maintain the colonic temperature at 42.0°C for 30, 20, 10, or 0 min, with the SAR varying from 0.8 to 6.6 W/kg. The time-averaged SARs were compared among groups to determine if concomitant 2ME exposure altered the SAR required to maintain colonic temperature at a specified level.

Teratology dose–effect relationships for 2ME and RF radiation

Specific concentrations of 2ME were prepared in distilled water (10 ml/kg). The solutions were verified by flame ionization gas chromatography to be within 10% of the target concentrations by the NIOSH Division of Physical Sciences and Engineering. Rats were gavaged with the specified dose of 2ME or distilled water immediately prior to preparing the animals for irradia-

tion or sham irradiation (preparation time of 5–10 min).

Dams were sacrificed on day 20, and fetuses were removed serially. Fetuses were blotted dry, weighed, and examined for malformations by trained observers who were blind to the treatment conditions of the fetuses at the time of observation. One-half of the fetuses were preserved in ethanol and were subsequently eviscerated, macerated in 1.5% KOH, stained with alizarin red-S, and examined for skeletal malformations and variations using a technique modified from Staples and Schnell ('63). The other half of the fetuses were preserved in Bouin's solution and were subsequently examined for visceral abnormalities using the Wilson ('65) technique.

In addition to the day 13 exposures, the present study also included exposure on day 9. Lary et al. ('82) demonstrated that RF radiation administered on gestation day 9 produced the highest incidence of malformations, as well as the most severe malformations. Administration of 2ME on gestation day 9 would also be expected to produce cardiovascular malformations in the fetuses. Since a higher incidence of, as well as a wider variety of, malformations would be expected from administration on gestation day 9, the present study included rats exposed on gestation day 9. A two-factor design was utilized, with 2ME and RF radiation each applied at five levels (see Table 1). Based on the results of the exploratory study (Nelson et al., '91a) and observations of Lary et al. ('83),[3] the following conditions were investigated in the present research, using six pregnant rats per cell. Expecting that day 9 exposures would potentially provide the greatest information as to interactions, we included a full design for day 9, and a partial design for day 13 exposures. The cells with two numbers (viz., 6/6) included rats exposed on gestation day 9 (6 rats/cell) and rats exposed on gestation day 13 (6 rats/cell). Cells with one number included rats exposed only on day 9.

Previous research suggested that 75 mg/kg 2ME alone, or a 10-min exposure to RF radiation at 42°C alone, would not produce detectable external malformations. It was not known if 75 mg/kg 2 ME or 10-min RF radiation exposure would produce malformations detectable using visceral or skeletal examinations, with or without combined exposures. Each of the 25 treatment combinations for gestation day 9, and the 16 treatment conditions for gestation day 13, was presented in random order. This procedure was repeated six times, each time with a new random order. Since mortalities were expected in approximately one third of the dams maintained for 30 min at 42°C, the groups which included animals exposed for 30 min were doubled in the randomization procedure to ensure 6 surviving animals per cell. In a small percentage of cases, the irradiation procedure did not elevate the colonic temperature to 42.0°C within 1 hr (likely due to differences in thermoregulatory ability). In these cases, the dams were replaced by other rats, and the litters not reaching the target temperature were not included in the statistical analyses.

[3]Observations that maintaining rectal temperatures of rats at 41.0°C for 2 h on gestation day 9 increased the incidence of skeletal malformations from approximately 1 to 6%.

TABLE 1. Two-factor study design used in present study

	Duration of RF radiation exposure sufficient to maintain colonic temperature at 42.0°C (min)				
	Sham	0[1]	10	20	30
2ME (mg/kg) (gavage)					
0	6/6	6/6	6/6	6/6	6/6
75	6/6	6	6	6	6/6
100	6/6	6	6	6	6/6
125	6/6	6	6	6	6/6
150	6/6	6/6	6/6	6/6	6/6

[1]This column includes rats whose colonic temperatures were elevated to 42°C (requiring approximately 30 min), but then the radiation was turned off immediately. Thus, the exposure was for "0" time at 42°C. This column is contrasted with sham-exposed controls shown in the first column.

Statistical analyses

Statistical analysis of data in interaction/synergism studies varies (see Berenbaum, '89; Goldstein et al., '90; and Chou and Rideout, '91). We employed two tools in analyzing the data. The first procedure, described in an earlier paper (Nelson et al., '91a), determines whether the effect of two teratogens administered together exceeds the additive effect of each applied separately. This procedure, which we shall refer to as the *Z*-test, tests for synergy for a particular treatment combination of one level of RF radiation and one level of 2ME. A second procedure uses the generalized estimating equations (GEE) approach (Zeger and Liang, '86; Ryan, '92) to characterize the response to the two teratogens over a range of values for the RF and 2ME.

The litter is the unit of response for the *Z*-test. For this test we compute

$$Z = \bar{Y} - (\bar{W} + \bar{X}) \Big/ \sqrt{\frac{S_w^2}{n_w} + \frac{S_x^2}{n_x} + \frac{S_y^2}{n_y}}. \qquad (1)$$

(Note: In the equation on page 627 in Nelson et al., '91a, the square root of the denominator was inadvertently omitted.)

W represents the percentage of fetuses malformed in a litter treated only with a given level of RF. *X* represents the percentage of malformed fetuses in a litter treated only with a given level of 2ME. *Y* represents

the percentage of malformed fetuses in a litter receiving the combined application of the same level of RF as applied for W and the same level of 2ME as applied for X. For example, if W was determined for rats receiving 30 min of RF and 0 mg/kg of 2ME and X was determined for a rat receiving sham RF and 150 mg/kg of 2ME, then Y would be measured for rats receiving 30 min of RF and 150 mg/kg of 2ME. We then test to see if $\overline{Y}$ significantly exceeds $(\overline{W}+\overline{X})$.

The second statistical procedure (the GEE approach) served three purposes. First, it was useful in further assessing whether there was an interaction between RF and 2ME. Second, as an exploratory tool, it helped identify those malformations and exposure times which would be most informative for future interactive studies. Third, it provided a means of developing a dose–response curve.

The advantage of using the GEE approach is that it permits observations from the same litter to be correlated. In using the GEE approach, we simply treated the fetuses within a litter as repeated measures of that litter. Comparable approaches, such as logistic regression, would require the dubious assumption that fetuses from the same litter are independent observations. The results from the GEE procedure, when used with a binary response, are, however, quite similar to those obtained with logistic regression. As with logistic regression, the experimental unit in the GEE approach is the individual fetus. The correlation structure in our analyses was assumed to be exchangeable, i.e., the correlation between different pairs of litter mates was assumed to be the same for all pairs. With the GEE approach, we fit a model as follows:

$$\begin{aligned} Y &= logit = log\,[(P)/(1-P)] \\ &= \beta_0 + \beta_1 C + \beta_2 R_a + \beta_3 R_e + \beta_4 C R_e \end{aligned} \quad (2)$$

where

P = predicted fraction malformed
C = level of 2ME
R_a = ramp time (time taken to reach a colonic temperature of 42°C, during which RF radiation is applied)
R_e = experimental exposure time of RF radiation maintaining the colonic temperature at 42°C (as opposed to ramp time)

The model served as a basis for assessing interaction in two ways. First, we tested to determine if β_4 differed significantly from 0. This assesses whether the interaction has a significant effect on the logit. Second, we fit the same model without the interaction term present, and then compared the observed number of malformations with the predicted number. Having the observed number of malformations exceed the predicted number would suggest a positive interaction or synergy. The predicted numbers were calculated using $P = 1/(1 + e^{-Y})$.

The GEE model used in the analysis also included terms (dummy variables) for the block effect. In conducting the experiment, all possible treatment combinations were each administered once in a randomized order and this set of treatments constituted a block. The entire study consisted of six blocks. Although block should not have affected the factors of interest in the study, blocks were included in the model to take into account any possible effects due to blocks.

An advantage that the GEE approach with a binomial response shares with logistic regression is that one may express the impact of an effect in terms of an odds ratio by simply taking the antilogarithm of the product of the coefficient and the difference between two exposure levels. The results are presented both in terms of coefficients (β_i) and in terms of the odds ratio. The odds ratios given in the tables are for one unit of change in whatever units were used (viz., mg/kg or duration of exposure). In some cases, this represents an extrapolation, such as when we used 1 hr of exposure for RF radiation, because observations were made for a maximum of one-half hour exposure.

We ran this basic model, using the statistical package SPIDA (Gebski et al., '92) for rats exposed on day 9 to the teratogens and, then, separately, for rats exposed on day 13. For each exposure day, we analyzed the data separately for all external malformations, then for all visceral malformations, then for all skeletal malformations, and, finally, for specific malformations (e.g., metacarpals, brain malformations). (In running the model, we had to rescale RF radiation exposure time to hours. Using the original units [minutes] resulted in singular matrices during SPIDA's calculations. A singular matrix is basically one with the equivalent of a row of zeros, and makes some matrix operations impossible. Sometimes, the problem can be circumvented by rescaling the variable, as proved to be the case here.)

We also investigated the effects of the exposure to the teratogens on fetal weights. The above model (2) was used with two modifications. First, the outcome (weight) for the GEE procedure was assumed to be normally distributed. In effect, this gives results analogous to those that would be obtained in linear regression. Second, the independent variable representing the level of 2ME was rescaled by dividing by 10, so that the units were 10 mg/kg. The rescaling was necessary because the GEE procedure would not run in SPIDA in the original units, apparently for a reason similar to that described above with respect to singular matrices.

RESULTS

Approximately 94% of the dams were pregnant in all treatment groups. No maternal toxicity was apparent from 2ME administration. In the groups in which RF radiation maintained the colonic temperature at 42.0°C for 30 min, 20% died within 1 to 2 days after the exposure. Otherwise, duration-dependent adverse maternal effects (e.g., lethargy for up to 1 hr) were noted

TABLE 2. Results of Z-test for external malformations for rat fetuses exposed to teratogens on day 13 of development[1]

Level of 2ME	Z-score	P-value (2-sided)
75	2.13189	0.03302
100	3.32233	0.00089
125	2.02572	0.04279
150	1.72839	0.08392

[1]The dependent variable is the percent of fetuses in a litter showing at least one malformation. The RF radiation was for the sham compared to fetuses receiving 30 min of exposure at 42°C. The 2ME was for 0 mg/kg compared to the level shown below.

TABLE 3. Results of Z-test for external malformations for rat fetuses exposed to teratogens on day 13 of development[1]

RF exposure time (min)	Z-score	P-value (2-sided)
0	2.37157	0.01771
10	4.86558	0.00000
20	0.82922	0.40698
30	1.72839	0.08392

[1]The dependent variable is percent of fetuses in a litter showing at least one malformation. The 2ME was for 0 compared to 150 mg/kg and the RF radiation was for the sham compared to the level of RF shown below.

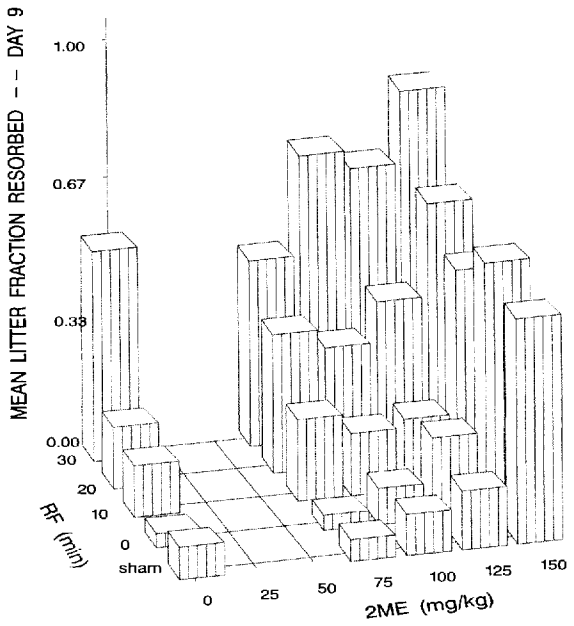

Fig. 1. Mean litter fraction of fetuses resorbed after exposure to the various combinations of 2ME and RF radiation on gestation day 9.

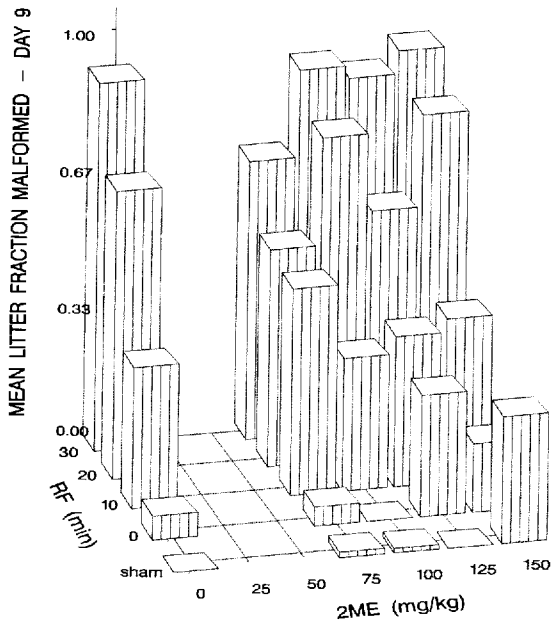

Fig. 2. Frequency of external malformations in rat fetuses after exposure to the various combinations of 2ME and RF radiation on gestation day 9; no apparent interaction is observed.

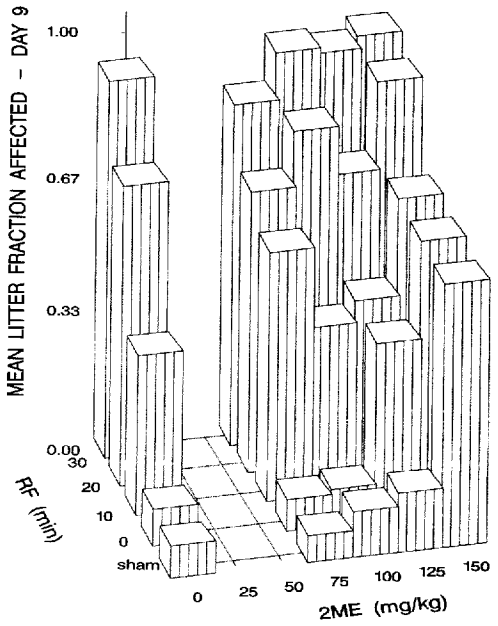

Fig. 3. Frequency of resorptions plus external malformations in rat fetuses after exposure to the various combinations of 2ME and RF radiation on gestation day 9; no apparent interaction is observed.

immediately following exposure to RF radiation, but prolonged adverse maternal effects were not observed. Mean (±SD) SARs ranged from approximately 6.5 (±0.3) W/kg for 0-min exposure to 5.3 (±0.5) W/kg for 30-min exposure. As expected, there were no significant differences in SAR between groups due to 2ME administration.

TABLE 4. Results of using GEE procedure for model given in equation (2) for rat fetuses exposed on day 9 of development[1]

Factor	Coefficient	*P*-value	Odds ratio	95% CI for O.R.
Constant	$\beta_0 = -4.47975$	0.00000		
2ME	$\beta_1 = 0.00820$	0.06171	1.0082	(0.9996, 1.0169)
Ramp time	$\beta_2 = 0.77027$	0.17080	2.1603	(0.7175, 6.5048)
RF	$\beta_3 = 9.60834$	0.00000	14888.3535	(436.2572, 508101.7500)
2ME × RF	$\beta_4 = -0.01337$	0.40242	0.9867	(0.9563, 1.0181)

[1]The response was the presence or absence of any external malformation. The correlation among littermates was estimated to be 0.2913.

TABLE 5. External malformations observed in rat fetuses exposed on day 9 of development[1]

Type of malformation	Total fetuses[2]	Number malformed	*P*-value for 2ME	Ramp time	RF	2 ME × RF
Agnathia	1705	92	0.47704	0.06160	0.00000	0.72574
Anophthalmia	1705	441	0.71750	0.06710	0.00000	0.71326
Cleft palate	1705	77	0.55597	0.04411 (+)	0.01490	0.65613
Encephalocoel	1696	44	0.25185	0.44818	0.05802	0.63382
Exencephaly	1696	202	0.07508	0.41706	0.00000	0.51208
Forepaw digits	1705	0[3]	—	—	—	—
Gastroscisis	1696	12[3]	—	—	—	—
Hindpaw digits	1705	3[3]	—	—	—	—
Hydrocephaly	1696	34	0.13766	0.83488	0.00220	0.50334
Imperforate anus	1696	3[3]	—	—	—	—
Micrognathia	1705	208	0.20498	0.02243 (+)	0.00003	0.55067
Microphthalmia	1705	75	0.01820 (+)	0.00756 (+)	0.45461	0.59894
Spina bifida	1696	2[3]	—	—	—	—
Tail	1705	17	0.0206 (+)	0.10785	0.02466	0.03314 (−)

[1]The number of fetuses exhibiting each specific malformation is given, as well as the *P*-values testing whether each of the coefficients in model (2) is equal to 0. Coefficients for the RF radiation were all positive. In cases where the *P*-value was less than 0.05 for the other factors, the sign of the coefficient is given in parentheses.
[2]Differences in numbers represent missing data.
[3]Too few malformations to run GEE procedure.

Results of *Z*-tests

The results of calculating the *Z*-statistic given in (1) for certain treatment combinations are presented in Tables 2 and 3. In Table 2, *W* was measured on rats receiving 30 min of RF radiation and 0 mg/kg of 2ME and *X* was measured on rats receiving RF sham and one of the levels of 2ME (75, 100, 125, or 150 mg/kg). *Y* was determined for rats receiving 30 min of RF radiation and the level of 2ME corresponding to that used for *X* (75, 100, 125, or 150 mg/kg).

In Table 3, *W* was determined for rats receiving 0 mg/kg of the 2ME and a given level of the RF (0, 10, 20, or 30 min). *X* was determined for rats receiving 150 mg/kg of the 2ME and the sham RF. *Y* was determined for rats receiving 150 mg/kg of 2ME and the level of RF corresponding to that used in determining *W*.

Results using GEE procedure

Day 9 exposures. Figure 1 presents the mean fraction of resorbed fetuses from day 9 exposure. External malformations were observed in surviving fetuses as follows (see also Fig. 2; Fig. 3 presents the fraction of fetuses resorbed or malformed). The fraction of pups with at least one malformation was 555/1695 = 0.3274. The results of the GEE procedure are presented in Table 4.

The only factor to have an unequivocal effect in the external malformations for day 9-exposed fetuses was the experimental time (viz., the time at the target temperature, as opposed to "ramp time" reaching that temperature) exposed to RF radiation. In addition to a very small *P*-value (actually less than 0.0000005) for the test, the estimated odds ratio for fetuses exposed to RF radiation compared to those not exposed was $e^{9.60834 \times 0.5} = 122$. This indicates that a fetus exposed to RF radiation at 42°C for 30 min would be 122 times more likely to have an external malformation than an unexposed fetus. As shown in Table 4, there is a suggestion of an effect by 2ME, but no effect due to ramp time or the interaction of 2ME and RF radiation. Some types of malformations were much more frequent than others, as shown in Table 5. Figure 4 shows the dose–response results for the most common malformations (agnathia, anophthalmia, exencephaly, and micrognathia).

The general pattern in the above malformations was that the effect of experimental RF radiation was highly significant for cases where the frequency of malforma-

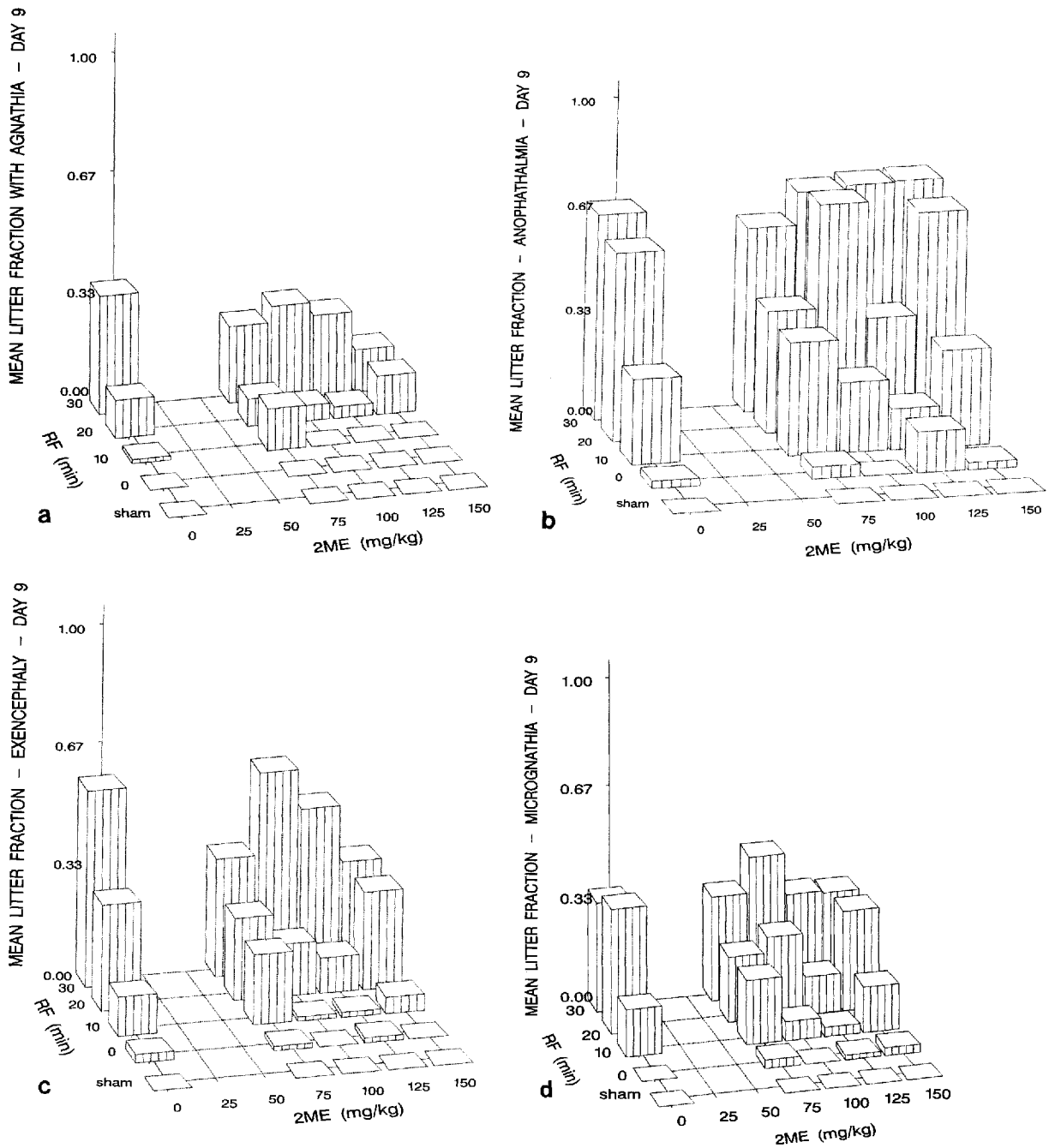

Fig. 4. Frequency of **(a)** agnathia, **(b)** anophthalmia, **(c)** exencephaly, and **(d)** micrognathia in rat fetuses after exposure to the various combinations of 2ME and RF radiation on gestation day 9; no apparent interaction is observed.

tions was relatively high, while the impact of other factors appeared to be low.

The results for visceral malformations observed in fetuses exposed to the teratogens on day 9 of development are presented in Table 6. The fraction of fetuses showing at least one malformation was 264/844 = 0.3128.

Again, RF radiation experimental time had a very significant effect, with a p-value of 0.000002 for the coefficient and an odds ratio of 5318 (for 30 min, odds ratio = 73). The frequencies of specific visceral malformations and p-values for the different factors are presented in Table 7.

Skeletal malformations seen in rat fetuses exposed

TABLE 6. Results of using GEE procedure for model given in equation (2) for rat fetuses exposed on day 9 of development[1]

Factor	Coefficient	*P*-value	Odds ratio	95% CI for O.R.
Constant	$\beta_0 = -4.53112$	0.00000		
2ME	$\beta_1 = 0.00971$	0.04886	1.0098	(1.0000, 1.0196)
Ramp time	$\beta_2 = 1.21842$	0.03521	3.3818	(1.0881, 10.5105)
RF	$\beta_3 = 8.57885$	0.00000	5317.9751	(151.7694, 186341.00)
2ME × RF	$\beta_4 = -0.01122$	0.50574	0.9888	(0.9567, 1.0221)

[1]The dependent variable is the presence or absence of any visceral malformation. The correlation among littermates was estimated to be 0.2763.

TABLE 7. Visceral malformations observed in rat fetuses exposed on day 9 of development[1]

Type of malformation	Total fetuses	Number malformed	*P*-value for 2ME	*P*-value for Ramp time	*P*-value for RF	*P*-value for 2ME × RF
Brain	844	201	0.13898	0.12667	0.00002	0.52472
Cleft palate	844	23[2]	—	—	—	—
Diaphragm	844	2[2]	—	—	—	—
Eyes	844	233	0.95075	0.01610	0.00008	0.42385
Heart	844	37	0.03502 (+)	0.67968	0.00075	0.04520 (−)
Stomach	844	7[2]	—	—	—	—
Vessels	844	7[2]	—	—	—	—

[1]The number of fetuses exhibiting each specific malformation is given, as well as the *P*-values testing whether each of the coefficients in model (2) is equal to 0. Coefficients for the RF radiation were all positive. The sign for other significant terms is given in parentheses.
[2]Too few malformations to run GEE procedure.

TABLE 8. Results of using GEE procedure for model given in equation (2) for rat fetuses exposed on day 9 of development[1]

Factor	Coefficient	*P*-value	Odds ratio	95% CI for O.R.
Constant	$\beta_0 = -3.77919$	0.00000		
2ME	$\beta_1 = 0.00920$	0.02354	1.0092	(1.0012, 1.0173)
Ramp time	$\beta_2 = 0.55022$	0.27439	1.7336	(0.6463, 4.6501)
RF	$\beta_3 = 10.33136$	0.00000	30679.8926	(577.15555, 1630852.87)
2ME × RF	$\beta_4 = -0.02172$	0.24746	0.9785	(0.9431, 1.0152)

[1]The dependent variable is the presence or absence of any skeletal malformation. The correlation among littermates was estimated to be 0.2246.

TABLE 9. Skeletal malformations observed in rat fetuses exposed on day 9 of development[1]

Type of malformation	Total fetuses	Number malformed	*P*-value for 2ME	*P*-value for Ramp time	*P*-value for RF	*P*-value for 2ME × RF
Forepaw digits	844	1[2]	—	—	—	—
Forepaw phalanges	844	1[2]	—	—	—	—
Hindpaw digits	844	3[2]	—	—	—	—
Hind limbs	844	5[2]	—	—	—	—
Hindpaw phalanges	844	3[2]	—	—	—	—
Metacarpals	844	1[2]	—	—	—	—
Metatarsals	844	3[2]	—	—	—	—
Ribs	844	98	0.34520	0.26059	0.00012	0.66792
Skull	844	259	0.76760	0.03006	0.00000	0.85895
Sterna centra	844	24[2]	—	—	—	—

[1]The number of fetuses exhibiting each specific malformation is given, as well as the *P*-values testing whether each of the coefficients in model (2) is equal to 0. In all cases where $P < 0.05$, the coefficients for the chemical, ramp time, and RF experimental time were positive.
[2]Too few malformations to run GEE procedure.

TABLE 10. Results of using GEE procedure for model given in equation (2) for rat fetuses exposed on day 9 of development[1]

Factor	Coefficient	*P*-value
Constant	$\beta_0 = 3.71847$	0.00000
2ME/10	$\beta_1 = -0.04207$	0.00000
Ramp time	$\beta_2 = 0.06925$	0.50965
RF	$\beta_3 = -1.68440$	0.00000
2ME/10 × RF	$\beta_4 = 0.06849$	0.01761

[1]The dependent variable is fetal weight and is assumed to be normally distributed. The correlation among littermates was estimated to be 0.4785.

on day 9 of development are presented in Table 8. At least one malformation appeared in 302/844 = 0.3578 of the fetuses.

Thus, the primary factor affecting skeletal malformations in fetuses exposed on day 9 is the experimental exposure to RF radiation. The results for specific malformations are shown in Table 9.

The effects on fetal weights are presented in Table 10, and graphically in Figure 5.

Clearly both 2ME and RF radiation had a highly significant effect on fetal weights. One way of looking at the effect of RF radiation is to observe that 0.5 hr of RF radiation changes the weight of a rat fetus on the average by (0.5)(−1.68440) = −0.84 g. Considering that the predicted weight for rat fetuses receiving no 2ME or RF radiation exposure would be 3.72 g (the value of the constant), the depression in fetal weights due to RF radiation is substantial (i.e., nearly 25%).

Day 13 exposures. The mean fraction of fetuses resorbed after exposure on gestation day 13 is presented in Figure 6. External malformations were observed in rat fetuses exposed to RF radiation and 2ME on day 13 of development as shown in Table 11 (see also Fig. 7; the sum of fetuses resorbed or malformed is shown in Fig. 8). The fraction of fetuses exhibiting at least one malformation was 658/1507 = 0.4366.

The odds ratio for 2ME pertains to increases of 1 mg/kg of 2ME. For an increase of 150 mg/kg (as in comparing the 0 mg/kg dose to the 150 mg/kg dose) the odds ratio would be $e^{150 \times 0.03701} = 257.62$. Thus, the probability of an external malformation appearing in a fetus exposed to 150 mg/kg of 2ME on day 13 is approximately 258 times greater than in a fetus not exposed

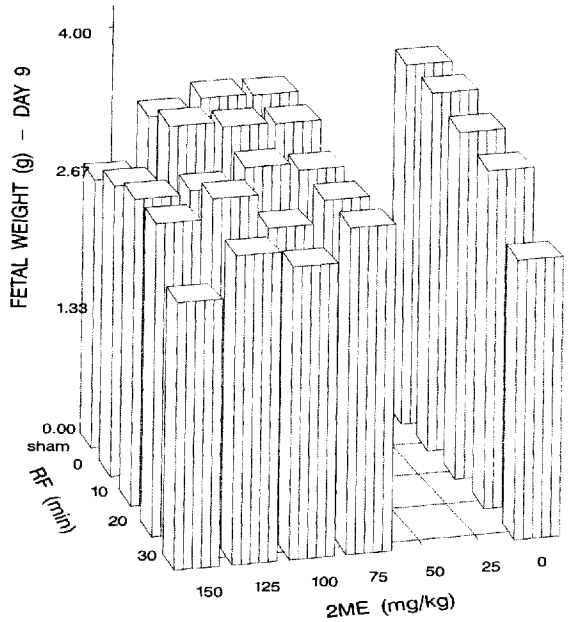

Fig. 5. Effect of exposure to the various combinations of 2ME and RF radiation on gestation day 9 on fetal weights. Note that the scales for both the *X* and *Y* axes are reversed to enable visualization of the data.

to 2ME. The results with regard to specific external malformations are presented in Table 12. Figures 9–11 present dose–response graphs of the most common malformations observed after day 13 exposure (forepaw, hindpaw, and tail malformations).

2ME and RF radiation (as well as ramp time) had significant impacts on only three external structures, namely the forepaw digits, the hindpaw digits, and the tail. 2ME affected the forepaw and hindpaw digits, but not the tail. RF radiation affected all three structures.

Visceral malformations were observed in rat fetuses exposed to teratogens on day 13 of development in very low numbers. The fraction of fetuses with at least one malformation was 8/723 = 0.0111. The GEE procedure did not run for the day 13 visceral malformations because the number of malformations was so low. The results with regard to specific malformations are presented in Table 13.

Skeletal malformations were present in rat fetuses exposed on day 13 of development as shown in Table

TABLE 11. Results of using GEE procedure for model given in equation (2) for rat fetuses exposed on day 13 of development[1]

Factor	Coefficient	*P*-value	Odds ratio	95% CI for O.R.
Constant	$\beta_0 = -7.45014$	0.00000		
2ME	$\beta_1 = 0.03701$	0.00000	1.0377	(1.0257, 1.0499)
Ramp time	$\beta_2 = 3.12196$	0.00000	22.6909	(6.3870, 80.6124)
RF	$\beta_3 = 10.62332$	0.00000	41081.9219	(735.4056, 2294957.25)
2ME × RF	$\beta_4 = -0.04155$	0.03507	0.9593	(0.9229, 0.9971)

[1]The dependent variable is the presence or absence of any external malformation. The correlation among littermates was estimated to be 0.2637.

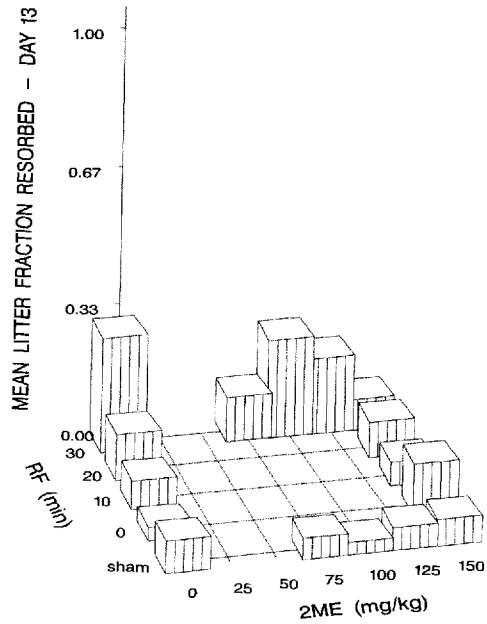

Fig. 6. Mean litter fraction of fetuses resorbed after exposure to the various combinations of 2ME and RF radiation on gestation day 13 (note the limited dose combinations administered on day 13 as described earlier in the paper).

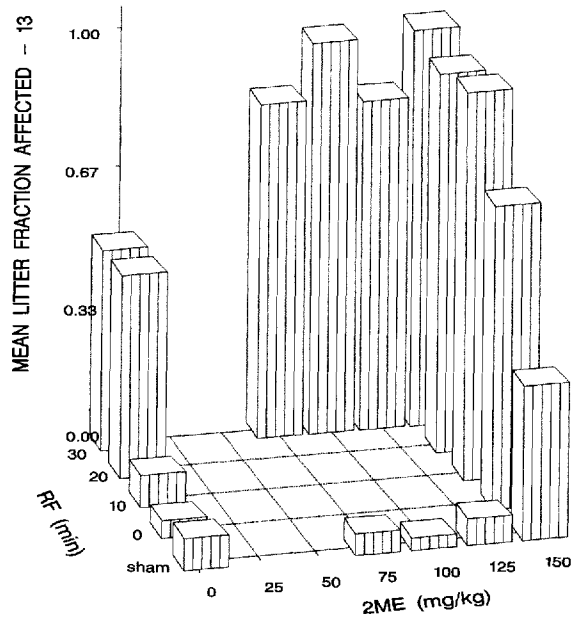

Fig. 8. Frequency of resorptions plus external malformations in rat fetuses after exposure to the various combinations of 2ME and RF radiation on gestation day 13; apparent interaction is observed.

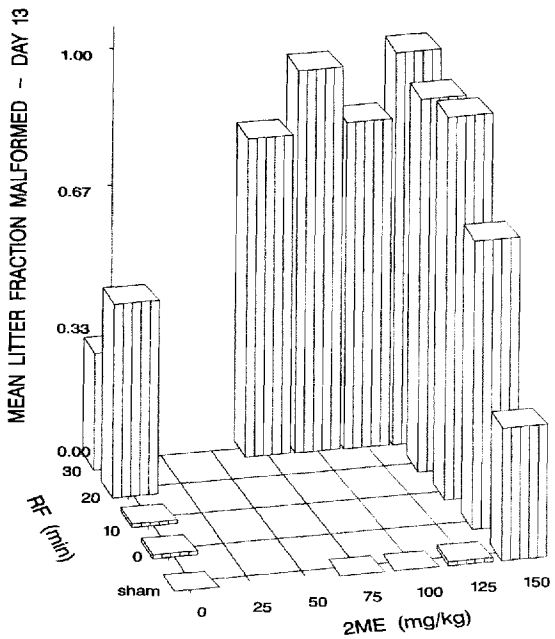

Fig. 7. Frequency of external malformations in rat fetuses after exposure to the various combinations of 2ME and RF radiation on gestation day 13. Apparent interaction is observed.

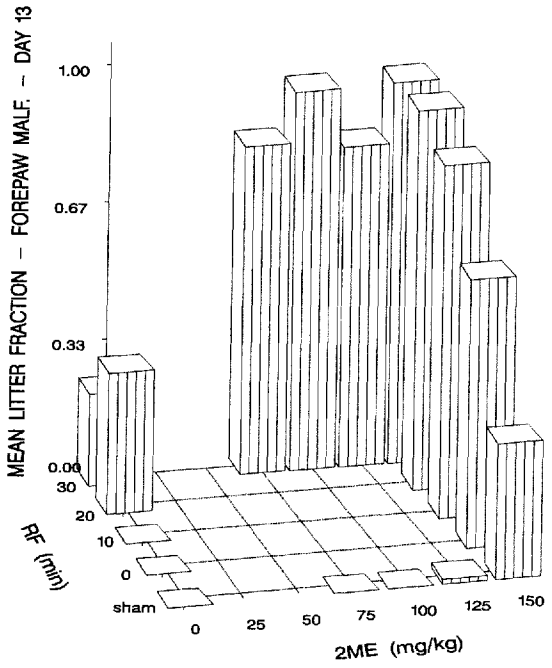

Fig. 9. Frequency of forepaw digit malformations in rat fetuses after exposure to the various combinations of 2ME and RF radiation on gestation day 13; apparent synergism is observed.

14. The fraction of fetuses showing at least one malformation was 410/740 = 0.5541.

Specific skeletal malformations are shown in Table 15.

The results of fetal weights after exposure on day 13 are presented in Table 16 and Figure 12. Again, as after exposure on day 9, the effects of the 2ME or RF radiation in depressing fetal weights were highly significant.

Table 17 presents the observed and predicted num-

TABLE 12. External malformations observed in rat fetuses exposed on day 13 of development[1]

Type of malformation	Total fetuses	Number malformed	*P*-value for 2ME	Ramp time	RF	2ME × RF
Agnathia	1509	1[2]	—	—	—	—
Anophthalmia	1509	0[2]	—	—	—	—
Cleft palate	1509	0[2]	—	—	—	—
Encephalocoel	1507	0[2]	—	—	—	—
Exencephaly	1507	1[2]	—	—	—	—
Forepaw digits	1509	632	0.00000	0.00001	0.00000	0.02397
Gastroscisis	1507	1[2]	—	—	—	—
Hindpaw digits	1509	273	0.00117	0.00029	0.00049	0.24765
Hydrocephaly	1507	0[2]	—	—	—	—
Imperforate anus	1507	1[2]	—	—	—	—
Micrognathia	1509	5[2]	—	—	—	—
Microphthalmia	1509	0[2]	—	—	—	—
Spina bifida	1507	0[2]	—	—	—	—
Tail	1509	294	0.44976	0.01479	0.00001	0.60381

[1]The number of fetuses exhibiting each specific malformation is given, as well as the *P*-values testing whether each of the coefficients in model (2) is equal to 0. Coefficients for the experimental RF radiation exposure, chemical, and ramp time were all positive. The coefficients for the interaction terms were negative.
[2]Too few malformations to run GEE procedure.

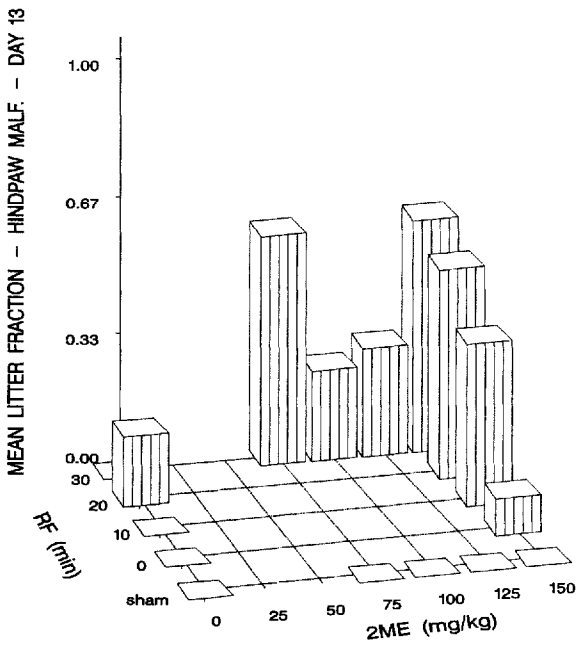

Fig. 10. Frequency of hindpaw digit malformations in rat fetuses after exposure to the various combinations of 2ME and RF radiation on gestation day 13; apparent synergism is observed.

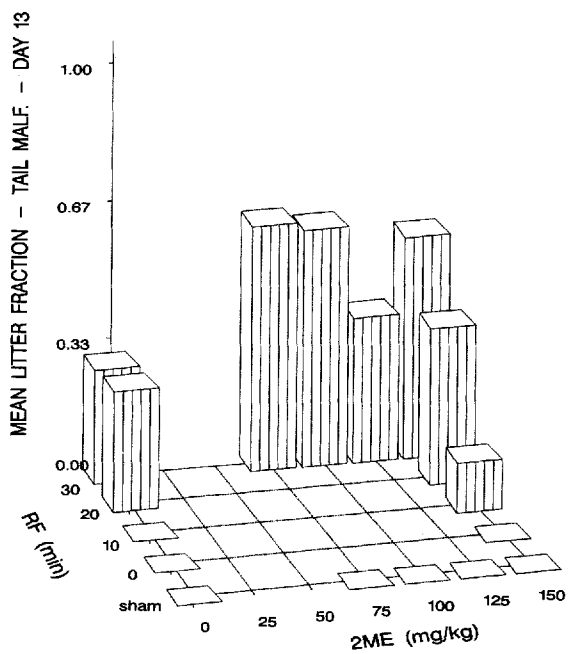

Fig. 11. Frequency of tail malformations in rat fetuses after exposure to the various combinations of 2ME and RF radiation on gestation day 13; no evidence of synergism is observed.

ber of fetuses with external malformations after day 13 exposures.

Dose–response curves can be generated using the equation $p = 1/(1 + e^{-Y})$ (see Methods). We generated these curves (Fig. 13) both with (Fig. 13a) and without (Fig. 13b) the ramp time present in order to provide some insight into the importance of ramp time, as well as the other two independent variables.

DISCUSSION

These data are consistent with, and extend, our previous results, and demonstrate increased developmental toxicity (i.e., increased malformations and decreased fetal weights) with increasing dosage of 2ME and duration of RF radiation. Our previous study had approximately 20 maternal animals per cell (in only four cells; Nelson et al., '91a). In contrast, the present

TABLE 13. Visceral malformations observed in rat fetuses exposed on day 13 of development

Type of malformation	Total fetuses	Number malformed	*P*-value for 2ME	Ramp time	RF	2ME × RF
Brain	723	2[1]	—	—	—	—
Cleft palate	723	2[1]	—	—	—	—
Diaphragm	723	0[1]	—	—	—	—
Eyes	723	0[1]	—	—	—	—
Heart	723	4[1]	—	—	—	—
Stomach	723	0[1]	—	—	—	—
Vessels	723	0[1]	—	—	—	—

[1]Too few malformations to run GEE procedure.

TABLE 14. Results of using GEE procedure for model given in equation (2) for rat fetuses exposed on day 13 of development[1]

Factor	Coefficient	*P*-value	Odds ratio	95% CI for O.R.
Constant	$\beta_0 = -5.23253$	0.00000		
2ME	$\beta_1 = 0.04544$	0.00000	1.0465	(1.0319, 1.0613)
Ramp time	$\beta_2 = 2.13899$	0.01477	8.4908	(1.5210, 47.4005)
RF	$\beta_3 = 7.94208$	0.00010	2813.2061	(50.9726, 155262.4688)
2ME × RF	$\beta_4 = -0.03847$	0.06467	0.9623	(0.9238, 1.0023)

[1]The dependent variable is the presence or absence of any skeletal malformation. The correlation among littermates was estimated to be 0.1525.

TABLE 15. Skeletal malformations observed in rat fetuses exposed on day 13 of development[1]

Type of malformation	Total fetuses	Number malformed	*P*-value for 2ME	Ramp time	RF	2ME × RF
Forepaw digits	740	294	0.00000	0.00000	0.00001	0.05524
Forepaw phalanges	740	298	0.00000	0.00000	0.00000	0.04741
Hindpaw digits	740	252	0.00000	0.00080	0.00001	0.03835
Hind limbs	740	220	0.00084	0.04058	0.01373	0.01171
Hindpaw phalanges	740	246	0.00000	0.00090	0.00001	0.01496
Metacarpals	740	262	0.00004	0.00000	0.00004	0.15503
Metatarsals	740	228	0.00000	0.00392	0.00000	0.02370
Ribs	740	27[2]	—	—	—	—
Skull	740	12[2]	—	—	—	—
Sterna centra	740	0[2]	—	—	—	—

[1]The number of fetuses exhibiting each specific malformation is given, as well as the *P*-values testing whether each of the coefficients in model (2) is equal to 0. All coefficients for 2ME, ramp time, and experimental RF time were positive, except for the ramp time of hind limbs. All coefficients for the interaction terms were negative.
[2]Too few malformations to run GEE procedure.

range-finding study was designed with only 6 animals per cell. One of the *Z*-tests in the present study confirmed the findings of Nelson et al. ('91a). With respect to the particular levels of RF radiation and 2ME used in the 1991 study (viz., RF = sham, 30 min; 2ME = 0, 150 mg/kg), the results for the current study were significant at $P = 0.04196$, as compared to $P = 0.02197$ for Nelson et al. ('91a).

The *Z*-tests generally indicate an effect greater than additivity for intermediate levels of the chemical (75, 100, and 125 mg/kg) when combined with 30 minutes of RF radiation exposure (Table 2). Figure 14 is a visual presentation of the fraction of fetuses malformed versus the fraction that would be expected based on an additive model of interaction. As can be seen, each of the doses of 2ME (75, 100, 125, and 150 mg/kg) produced effects greater than additivity for 30 min exposure to RF radiation. There was also evidence of synergism with intermediate levels of RF radiation (0 and 10 min) when combined with 150 mg/kg of 2ME (Table 3).

The results of the *Z*-tests suggest that synergy is evident with RF radiation elevating colonic temperatures for as low as 10 min and 2ME below 125 mg/kg. Another set of treatment combinations needing investigation as suggested from the data would be with 2ME absent or present at 100 mg/kg (cf. Figs. 9 and 12) and RF radiation ranging from 0 to 10 min (or, as a practical matter, with lower temperatures since durations shorter than 5 or 10 min intervals may be biologically

TABLE 16. Results of using GEE procedure for model given in equation (2) for rat fetuses exposed on day 13 of development[1]

Factor	Coefficient	*P*-value
Constant	$\beta_0 = 3.83514$	0.00000
2ME/10	$\beta_1 = -0.04787$	0.00000
Ramp time	$\beta_2 = -0.13183$	0.31956
RF	$\beta_3 = -1.35470$	0.00080
2ME × RF	$\beta_4 = 0.04190$	0.25994

[1]The dependent variable is fetal weight and is assumed to be normally distributed. The correlation among littermates was estimated to be 0.6847.

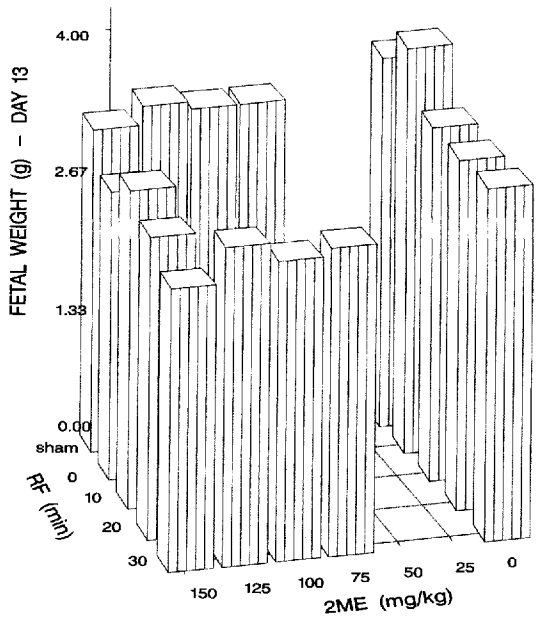

Fig. 12. Effect of exposure to the various combinations of 2ME and RF radiation on gestation day 13 on fetal weights. Note that the scales for both the *X* and *Y* axes are reversed to enable visualization of the data.

implausible). Ongoing studies are addressing these research needs.

As expected due to differences in organogenesis, a clear pattern emerged that exposure to teratogens on days 9 and 13 generally led to malformations in different structures. For example, the most frequent external malformations in the day 9 group were anophthalmia, exencephaly, and micrognathia (Table 5), whereas these malformations were rare or absent in the day 13 exposure group (Table 12). For the day 13 group, malformed forepaw digits, hindpaw digits, and tails were the most common external malformations (Table 12). These types of malformations were rare to nonexistent in the day 9 group (Table 5).

Such patterns of malformations would be important to take into account in future studies investigating synergistic effects of 2ME and RF radiation. As shown in Table 5 and Figure 4, 2ME did not appear to affect the occurrence of anophthalmia, micrognathia, or exencephaly. Looking for synergy between RF radiation and 2ME with respect to anophthalmia or micrognathia in rats exposed on day 9 would not appear to be a fruitful approach. On the other hand, Table 12 and Figures 9 and 10 show that, for the rats exposed on day 13, both the forepaw digits and hindpaw digits were strongly affected by both 2ME and RF radiation. Thus, for fetuses exposed to 2ME and RF radiation on day 13, forepaw and hindpaw digits would be useful indicators

TABLE 17. Predicted and observed number of malformations for day 13 external malformations[1]

RF/2ME	0	75	100	125	150
30					
Observed	15	67	88	62	135
Predicted	14.7	59.1	77.0	69.4	131.9
(Obs.−pred.)	**0.3**	**7.9**	**11**	**−7.4**	**3.1**
20					
Observed	36				85
Predicted	13.4				82.8
(Obs.−pred.)	**22.6**				**2.2**
10 Observed	1				84
Predicted	7.2				73.3
(Obs.−pred.)	**−6.2**				**10.7**
0					
Observed	1				54
Predicted	3.3				41.9
(Obs.−pred.)	**−2.3**				**12.1**
Sham					
Observed	0	0	0	1	29
Predicted	0.3	1.7	3.2	5.6	10.1
(Obs.−pred.)	**−0.3**	**−1.7**	**−3.2**	**−4.6**	**18.9**

[1]The predicted number are from a GEE model with no interaction term. For this model the coefficients were $\beta_0 = -5.877$, $\beta_1 = 0.025$, $\beta_2 = 2.926$, $\beta_3 = 6.266$.

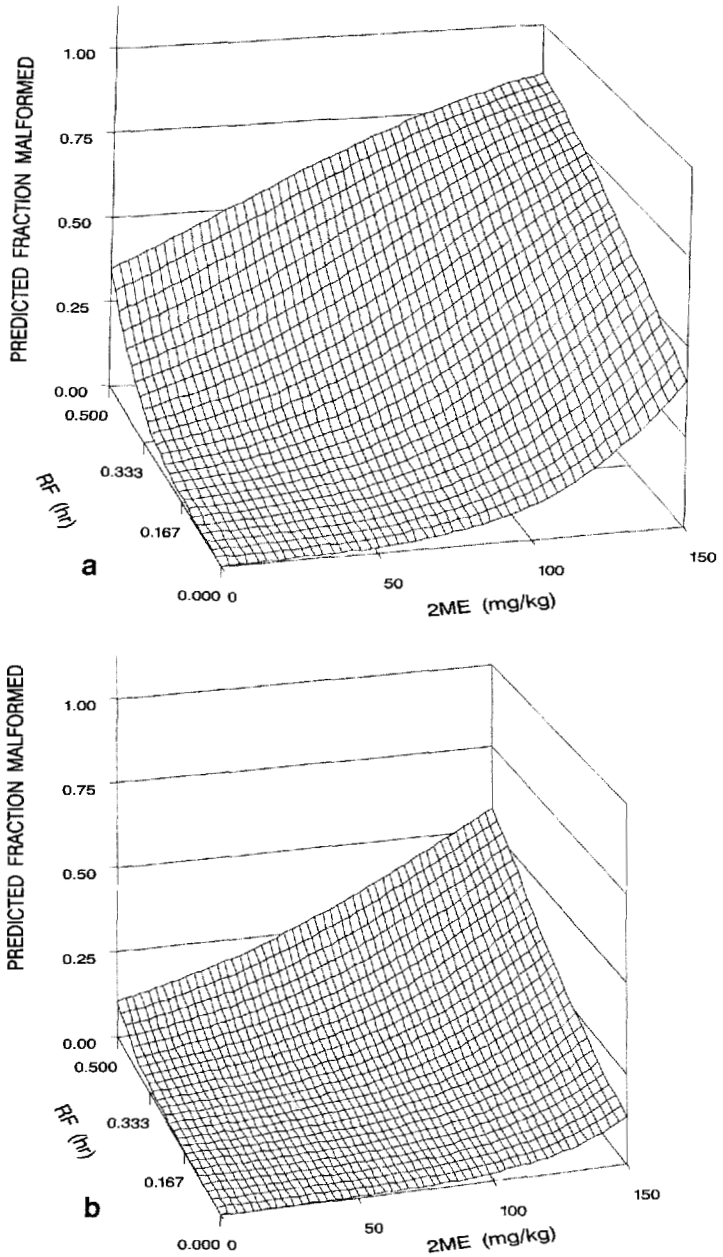

Fig. 13. Dose–response curves were generated using the equation $p = 1/(1+ e^{-Y})$ (see Methods) with Y defined as in (2). The values for the coefficients were taken from Table 11 with $\beta_0 = -7.450$, $\beta_1 = 0.037$, $\beta_2 = 3.122$, $\beta_3 = 10.623$, and $\beta_4 = -0.042$. In **(a)** all terms are included in predicting the response, using the average ramp time (0.483 hr) for all fetuses. In **(b)** all terms were included except ramp time.

of teratogenic interactions. Tail malformations for rats exposed on day 13 may not be as useful because they did not appear to be affected by 2ME (Fig. 11).

In the rats examined for skeletal malformations, a similar difference was seen between exposures on day 9 and day 13. As shown in Tables 9 and 15, the two most common types of malformations for the day 9 group (in skulls and ribs) were both uncommon in the day 13 group. Skull and rib malformations in rats exposed on day 9 may not be useful indicators of teratogenicity in a synergy study of 2ME and RF radiation, however, because they were not significantly affected by 2ME. The most common types of malformations in the day 13 group (forepaw digits, forepaw phalanges, hindpaw digits, hind limbs, hindpaw phalanges, metacarpals, and metatarsals) were rare in the day 9 group. All of the common malformations in the day 13 group showed highly significant effects from both 2ME and RF radi-

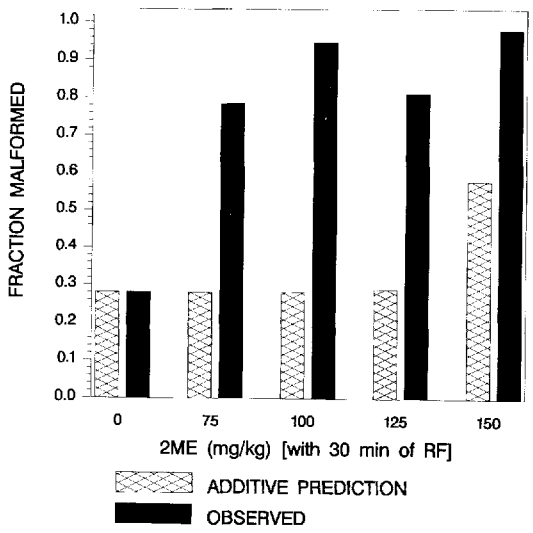

Fig. 14. Frequency of observed versus expected malformations in rat fetuses after administration of 0, 75, 100, 125, or 150 mg/kg 2ME along with 30 min of RF radiation exposure maintaining colonic temperatures at 42°C. Apparent synergism is observed at 75, 100, 125, and 150 mg/kg.

ation, and would thus be appropriate indicators of teratogenicity in interaction studies.

The skeletal malformation data (Table 15) thus corroborate the findings for the external malformation data (Table 12) with respect to forepaw digits and hindpaw digits. These data showed, in addition, that the forepaw phalanges, hindpaw phalanges, hind limbs, metacarpals, and metatarsals would probably be suitable indicators of teratogenicity in interaction studies of 2ME and RF radiation.

The visceral malformations also exhibited differences between day 9 and day 13 exposures. As seen in Tables 7 and 13, malformations of the brain and eyes were much more common in the fetuses exposed on day 9 than those exposed on day 13. The foregoing results, therefore, suggest that skeletal and visceral examinations added little information to the teratogenic interactions. Consequently, future research will focus on the external malformations produced after exposures on gestation day 13.

Testing of the interaction term from the GEE procedure provided no evidence that interaction between RF radiation and 2ME increased the logit of the response. On the other hand, a comparison of observed and predicted malformations from the GEE model with no interaction term did provide evidence for a positive interaction between RF and 2ME (see Table 17). In six of the seven treatment combinations where an enhanced response could have occurred, it did. In the one treatment combination where it did not (viz., Table 17, RF 30 and 2ME 125), 14 of the 16 normal fetuses were from 1 female rat, and all of fetuses from this litter had unusually high weight. If this unusual litter had not been present, the observed malformations would have substantially exceeded the predicted number for this treatment combination, too.

Whether or not one views the GEE analysis as providing evidence for a positive interaction or synergy depends on how one defines synergy. A recent review by Nelson ('94) recommends that synergism be defined as a response that is significantly greater than the sum of the effects of each agent administered alone. Under this definition, the response surface obtained with the GEE provides evidence of synergism for those areas where the surface exceeds a linear additive relationship between the fraction malformed in the combined exposure conditions and the independent conditions (viz., RF radiation and 2ME alone).

The impact of ramp time has implications for future research with regard to choice of indicators of teratogenicity. When malformations were pooled in the day 9 exposure group, only the visceral malformations showed an effect due to ramp time ($P = .035$, Table 6). In contrast, the day 13 external malformations showed a highly significant ramp time effect ($P = 0.00000$, Table 11; see also Fig. 13b, in which ramp time was not included in the model), as did certain skeletal structures (Table 15). Specific structures showing a particularly strong ramp time effect for the day 13 fetuses included (Table 15) forepaw digits ($P = 0.00000$), forepaw phalanges ($P = 0.00000$), and metacarpals ($P = 0.00000$). Determining which structures exhibit ramp time effects would be important for future studies conducted at lower temperatures, because, by definition, a ramp time effect is an effect due to RF radiation producing a colonic temperature rise from baseline, but not reaching 42°C. Thus the forepaw digits, forepaw phalanges, and metacarpals, as well as the hind digits and hind phalanges, would likely be good candidates for indicators of teratogenicity for studies using RF radiation producing colonic temperatures less than 42°C. Figure 13 illustrates the difference in response surface graphs when including ramp time or not including ramp time.

Although the present experiments utilized a relatively high exposure to RF radiation, the data of Marcickiewicz et al. ('86) suggest that lower levels of irradiation may produce significant enhancement of developmental toxicity. They found that even low-level exposures to 2450 MHz RF radiation (10 mW/cm^2, 4–5 W/k, no measurable temperature rise) produced dramatic increases in the teratogenicity of cytosine arabinoside in mice. Of course 2ME may act by a totally different mechanism than does cytosine arabinoside, so the relevance of that study to the present one is uncertain.

Our data suggest that the threshold for interactive effects lies below 75 mg/kg. Figure 13 illustrates the dose–response curves for interactive effects (although the intermediate levels represent interpolation from higher dose data points). Ongoing research is addressing the threshold for interactive effects.

Additional ongoing research is addressing a potential mechanism of action: if RF radiation affects the metabolism of 2ME. Future research will address the ability of 10 MHz RF radiation at levels which produce no measurable hyperthermia to enhance the teratogenicity of 2ME in rats. This research defining the lowest effect levels of interaction between 2ME and RF radiation will facilitate a more accurate risk assessment of worker exposure which could potentially be used in setting exposure standards for these two agents, and may have implications for risk assessment with concurrent exposures to other agents as well. It is clear that organizations involved in risk assessment and standard-setting must begin to consider interactive effects of combined chemical and physical agent exposures if personnel exposure standards are to be comprehensive in addressing realistic occupational health risks.

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