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Developmental Toxicity of a Commercial Herbicide Mixture in Mice: I. Effects on Embryo Implantation and Litter Size

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We investigated the developmental toxicity in mice of a common commercial formulation of herbicide containing a mixture of 2,4-dichlorophenoxyacetic acid (2,4-D), mecoprop, dicamba, and inactive ingredients. Pregnant mice were exposed to one of four different doses of the herbicide mixture diluted in their drinking water, either during preimplantation and organogenesis or only during organogenesis. Litter size, birth weight, and crown–rump length were determined at birth, and pups were allowed to lactate and grow without additional herbicide exposure so that they could be subjected to additional immune, endocrine, and behavioral studies, the results of which will be reported in a separate article. At weaning, dams were sacrificed, and the number of implantation sites was determined. The data, although apparently influenced by season, showed an inverted or U-shaped dose–response pattern for reduced litter size, with the low end of the dose range producing the greatest decrease in the number of live pups born. The decrease in litter size was associated with a decrease in the number of implantation sites, but only at very low and low environmentally relevant doses. Fetotoxicity, as evidenced by a decrease in weight and crown–rump length of the newborn pups or embryo resorption, was not significantly different in the herbicide-treated litters. *Key words:* **2,4-D, developmental toxicity, dicamba, embryo implantation, fetal loss, herbicide mixtures, mecoprop.** *Environ Health Perspect* **110:1081–1085 (2002). [Online 12 September 2002]** *http://ehpnet1.niehs.nih.gov/docs/2002/110p1081-1085cavieres/abstract.html*

Although they are not conclusive, a number of epidemiologic studies have linked pesticide exposure to reproductive and developmental toxicity in humans. Pastore et al. (1997) showed a clear positive association between occupational exposure to pesticides, especially during early pregnancy, and the risk of stillbirths in California, and Kristensen et al. (1997), associated central nervous system and limb defects with parental use of pesticide spraying equipment in Norway. In contrast, a case–control study in Holland determined little effect of pesticide exposure on the incidence of central nervous system defects in children of mothers involved in agricultural activities (Blatter et al. 1996); environmental pollution with pesticides, regardless of the occupation of the mother, could have explained the increased risk of spina bifida found in this study. Similarly, Shaw et al. (1999) did not find a clear pattern of association between specific pesticide exposures and risk for birth defects.

More recently, Bell et al. (2001) conducted a case–control study in California, where they linked a statewide database of restricted pesticide applications to residence of the mother to estimate daily pesticide exposure status. The data showed that risk of fetal death from congenital anomalies was increased with maternal pesticide exposure occurring during weeks 3–8 of pregnancy. Additionally, they showed that the odds ratio for pesticide exposure causing fetal death increased when the exposure occurred within the same square mile of maternal residence.

Wives of Dutch fruit growers exposed to pesticides showed an increase in time to pregnancy, defined as the number of noncontraceptive menstrual cycles or months required for a couple to conceive, although no specific pesticide or group of pesticides could be singled out as responsible for the effect (de Cock et al. 1994). Time to pregnancy was also used by Curtis et al. (1999), but they did not find a consistent pattern of association between pesticide exposure and time to pregnancy among Canadian farm couples. However, Curtis et al. did show that some specific pesticides such as organophosphate insecticides and phenoxyacid herbicides were associated with a decrease in fecundity when women engaged in pesticide-related activities.

Phenoxyacid and benzoic acid-derived herbicides are currently used in more than 1,500 different commercial formulations, including those that are commonly used at home (Kamrin 1997); thus there is an urgent need to explore in detail the potential reproductive and developmental toxicity of these products. Earlier studies have assessed the teratogenic potential of individual compounds at high doses that we now consider only to be toxicologically relevant. We modified a U.S. Environmental Protection Agency (EPA) Segment II developmental toxicity study (U.S. EPA 2000) protocol to include a low, environmentally relevant dose to test whether *in utero* exposure to a herbicide mixture containing the phenoxyacid derivatives 2,4-D and mecoprop, the benzoic acid derivative dicamba, and other inactive ingredients leads to developmental toxicity and/or developmental immune and endocrine defects in juvenile mice.

Here we report the dose-dependent effect of the herbicide mixture on litter size and number of implantation sites; effects on immune, endocrine, and behavioral parameters will be reported in a separate article. We also report on the effect of exposure period (preimplantation and organogenesis versus organogenesis) and seasonal influences on the responses.

Materials and Methods

A commercial herbicide product containing 2,4-dichlorophenoxyacetic acid (2,4-D) (7.59%), mecoprop (3.66%), dicamba (0.84%), and inert ingredients (87.91%) was diluted in the drinking water at the four dose levels indicated in Table 1. The very low dose corresponds to the reference dose (RfD) for 2,4-D, 0.01 mg/kg/day, and is lower than its maximum contaminant level (MCL), 0.07 ppm, as established by the U.S. EPA drinking water standards and health advisories (U.S. EPA 2002). Concentrations were confirmed by gas-liquid chromatography at the Wisconsin Department of Agriculture, Trade and Consumer Protection Bureau of Laboratory Services, and were always within ± 20% of the target dose.

All experiments were conducted in the animal facilities of the Department of Zoology at the University of Wisconsin-Madison using ND4 mice from Harlan Sprague Dawley (Indianapolis, IN). Two weeks before the start of each experiment, 6-week-old mice were purchased and housed in

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the same rooms where the experiments were conducted, allowing them to adjust to the light–dark cycle and temperature of the animal rooms. The mice were then mated at 8 weeks of age and checked daily for copulatory plugs. After mating, we removed the males and discarded them; pregnant females were maintained in hanging stainless-steel cages until after parturition. Animals had free access to food and water. Animal feed was certified by the manufacturer to be pesticide-free (Lab Diet 5002; PMI Nutrition International Inc., Brentwood, MO). The herbicide-containing drinking water was delivered in aluminum-foil–covered glass water bottles during either preimplantation and organogenesis [gestation days (GD) 0–15] or only organogenesis (GD 6–15), with GD0 being determined by the presence of a vaginal sperm plug in the mated female.

The cages were fitted with a stainless-steel box that allowed free access to water via a small hole in one side while keeping the mice away from the bottle spout. The box received any water dripping from the water bottle. The bottom of the box was covered with a layer of mineral oil (Sigma, St. Louis, MO) to stop any leaked water from evaporating. We weighed water bottles and oil boxes daily to obtain a more exact determination of the dose of herbicide the animals received. We determined water consumption and thus herbicide doses from the difference in the weights of the bottles and boxes on 2 consecutive days.

We recorded weights of pregnant females on GD0, GD6, GD9, GD12, GD15, and GD18. Maternal observations such as changes in food and water consumption, behavior and activity, and the presence of toxicity signs were also made to ensure that pregnancy was proceeding normally. At parturition, we checked each litter for total number of live pups. Occasional dead pups or cannibalized pups were not included in litter size. We individually weighed each pup and measured its crown–rump length using a graduated ruler. Once the data were recorded, the litter of newborns was culled to 8 pups to ensure a homogeneous growth of the litter. The 8 remaining pups were kept with the mother until weaning at 3 weeks (postnatal day 21) and then were allowed to grow until week 6 for additional immune, endocrine, and behavioral assays.

We determined the number of implantation sites by staining the uterine horns with a solution of 10% ammonium sulfide (Sigma) according to Salewski (1964). Females were killed by cervical dislocation after ether exposure; the uterine horns were dissected, rinsed, and kept in phosphate-buffered saline in plastic petri dishes to prevent drying. The dissected uteri were stained for 10 min with a few drops of the ammonium sulfide solution, after which we counted implantation sites, appearing as dark rings.

We used SuperANOVA software (SAS Institute, Cary, NC) for statistical analysis. Statistical advice was also given by the Statistical Consulting Service of the College of Agriculture and Life Sciences at the University of Wisconsin-Madison. Statistical tests used were analysis of variance (ANOVA) for litter size, analysis of covariance (ANCOVA) using litter size as a covariate for newborn weight and crown–rump length, implantation sites, and resorptions, and repeated-measures ANCOVA for weight changes during pregnancy, where weight of the mother at GD6, 9, 12, 15, and 18 was the repeated measure and weight at GD0 was the covariate.

We conducted all experiments in accordance with laboratory animal use and care protocols as established by the Research Animal Resource Center, University of Wisconsin-Madison.

Results

The data presented are the result of a series of experiments performed over a period of 2 years. We statistically analyzed data from control animals from different experiments to ensure minimal interexperiment variability. Similarly, the amount of water consumed by the mice in the different experiments was also analyzed to test for homogeneous water consumption in different experiments and dose levels. The objectives of this analysis were 2-fold. On one hand, it was necessary to show that mice from different experiments drank the same amount of water and thus received the same level of herbicide mixture. On the

Table 1. Concentration of herbicides in drinking water (ppm) and dose (mg/kg/day) of herbicide mixture administered to mice.

	2,4-D		Mecoprop		Dicamba	
Dose	ppm	mg/kg/day	ppm	mg/kg/day	ppm	mg/kg/day
Very low	0.039	0.01	0.019	0.004	0.004	0.0009
Low	0.32	0.1	0.15	0.040	0.035	0.009
Intermediate	77	20	36.7	8.07	8	1.83
High	400	100	200	40.39	42.4	9.166

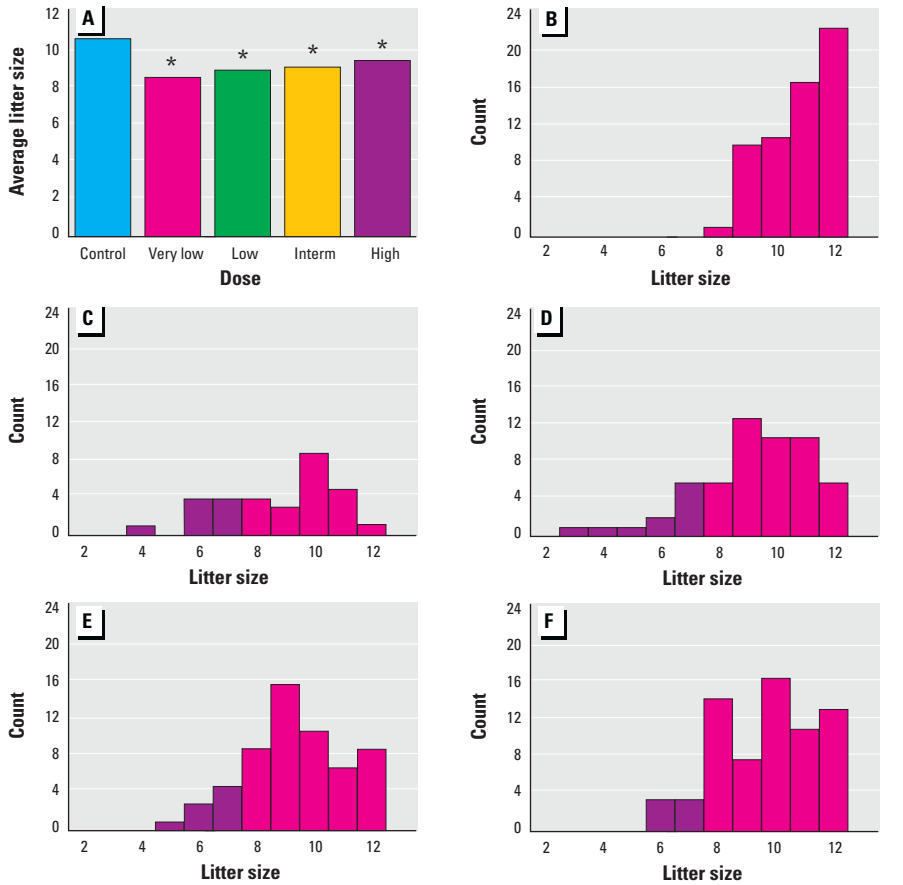

Figure 1. (*A*) Litter size after exposure to herbicide mixture; summary of groups ($n = 275$). Distribution of litter size in each dosing group: (*B*) control ($n = 62$); (*C*) very low dose ($n = 31$); (*D*) low dose ($n = 58$); (*E*) intermediate dose ($n = 61$); (*F*) high dose ($n = 63$). Interm, intermediate.
*$p < 0.05$, ANOVA, Bonferroni.

other hand, it was also necessary to show that mice, especially those assigned to different dose levels, did not differ in their water intake, so that amount of water ingested could be ruled out as a factor affecting the outcome of the experiment. In both cases—analysis of data from control animals and analysis of water consumption—we found no statistically significant differences, thereby indicating a homogeneous response across experiments (data not shown).

Figure 1A shows the effects of herbicide exposure on litter size. There was a significant effect of dose on litter size (ANOVA, $p < 0.001$) such that herbicide administration caused a decrease in the number of live-born pups at all dosage levels (Bonferroni *post hoc* test, $p < 0.05$). Figure 1B–F shows the distribution of litter sizes within each test group. Notice that each herbicide dose group has smaller litters than the smallest litter in the control group.

Table 2 shows the combined effect of season and dose on litter size after exposure to the herbicide mixture. All doses caused a decrease of approximately 20% in the number of pups born, although the response varied from season to season. During the fall, a significant decrease ($p = 0.0005$) in litter size was observed at the high dose, whereas in winter, the very low and intermediate dose produced this effect ($p = 0.0016$ and 0.0020, respectively). During spring, the statistically significant decrease in litter size occurred at the low ($p = 0.0051$) and in the summer at the low ($p = 0.0032$) doses, and the very low ($p = 0.0003$) doses. Note that period of administration during spring included both the preimplantation and the organogenesis period, whereas administration in all other seasons was only during organogenesis. The different exposure periods did not seem to cause a difference in the response.

There was a significant effect of pesticide dose on both newborn weight (ANCOVA, $p = 0.061$) and on crown–rump length (ANCOVA, $p = 0.002$). As expected, newborns from smaller litters weighed more and had longer crown–rump lengths than mice from other litters (Table 3), and mothers producing smaller litter sizes gained less weight during pregnancy, specially after GD12 (data not shown).

Figure 2 shows the effect of pesticide dose on implantation sites, litter size, and embryo resorptions. The data in Figure 2 are a subset of the data in Figure 1 because implantation sites were not done on all mice. Only mice with implantation data were used to calculate the litter sizes in Figure 2. Resorptions were determined from the difference between implantation sites and litter size in this figure. Litter size and implantation sites were significantly affected by dose (ANOVA, $p < 0.001$ and $p = 0.004$, respectively), but resorptions were not significantly affected (ANOVA, $p = 0.383$). Note that implantation sites and litter size in the very low and low doses both differed significantly from their control and high doses, respectively (Bonferroni test, $p < 0.05$).

Table 4 shows the combined effect of season and dose on the average number of implantation sites and resorptions per mouse after herbicide exposure. No effects due to the herbicide mixture were observed at the higher doses. Although not always statistically significant, the very low and the low doses produced a decrease in the number of implantation sites. During spring the number of resorptions as well as the number of implantation sites was decreased compared to the controls, but the trend was not significant.

Table 2. Litter size in females exposed to a commercial mixture of 2,4-D, mecoprop, and dicamba during pregnancy in different seasons [mean ± SD (*n*)].

Dose	Fall	Winter	Spring	Summer
Control	11.4 ± 1.3 (18)	10.8 ± 1.1 (17)	11.0 ± 1.5 (15)	10.6 ± 1.5 (12)
Very low	ND	9.2 ± 1.8 (19)**	ND	8.2 ± 2.2 (12)**
Low	10.5 ± 1.5 (14)	9.4 ± 1.4 (11)	9.4 ± 1.9 (18)**	7.7 ± 2.3 (15)*
Intermediate	9.4 ± 1.5 (18)	8.9 ± 2.0 (14)*	9.5 ± 1.9 (17)	9.3 ± 1.6 (12)
High	8.8 ± 1.6 (18)*	9.9 ± 1.9 (13)	10.3 ± 1.9 (19)	9.9 ± 1.0 (13)

ND, not determined. Pregnant mice were exposed during organogenesis (GD5–15) except for the spring experiment, when they were exposed during preimplantation and organogenesis (GD0–15).
*$p < 0.05$; **$p < 0.01$, ANCOVA, Bonferroni.

Table 3. Newborn weight and crown–rump length after *in utero* exposure to a commercial mixture of 2,4-D, mecoprop, and dicamba during pregnancy in different seasons (mean ± SD).

	Fall	Winter	Spring	Summer
Weight (g)				
Control	1.36 ± 0.11 (18)	1.43 ± 0.15 (17)	1.46 ± 0.19 (15)	1.34 ± 0.07 (12)
Very low	ND	1.51 ± 0.16 (19)	ND	1.48 ± 0.14 (12)
Low	1.41 ± 0.14 (14)	1.49 ± 0.14 (11)	1.51 ± 0.16 (18)	1.46 ± 0.17 (15)
Intermediate	1.39 ± 0.13 (18)	1.47 ± 0.17 (14)	1.49 ± 0.17 (17)	1.44 ± 0.12 (12)
High	1.41 ± 0.12 (18)	1.47 ± 0.11 (13)	1.41 ± 0.16 (19)	1.31 ± 0.1 (13)
Crown–rump length (mm)				
Control	26.2 ± 0.6 (18)	26.9 ± 0.6 (17)	27.0 ± 0.5 (15)	27.3 ± 0.6 (12)
Very low	ND	26.8 ± 1.0 (19)	ND	27.4 ± 0.3 (12)
Low	26.4 ± 1.0 (14)	27.0 ± 0.4 (11)	27.1 ± 0.9 (18)	27.8 ± 0.9 (15)
Intermediate	26.3 ± 0.5 (18)	27.2 ± 0.5 (14)	27.0 ± 0.1 (17)	27.5 ± 0.7 (12)
High	26.4 ± 0.5 (18)	26.8 ± 0.9 (13)	26.7 ± 0.7 (19)	27.0 ± 0.5 (13)

ND, not determined. Pregnant mice were exposed during organogenesis (GD5–15), except for the spring experiment, when they were exposed during preimplantation and organogenesis (GD0–15);

Discussion

No reports on the reproductive or developmental toxicity of mecoprop or dicamba have been published in the recent literature, although dicamba has been reported to be embryotoxic to mallard duck embryos (Hoffman and Albers 1984). Early developmental 2,4-D toxicity studies seem to indicate that malformations only occur at high doses of exposure and are mainly related to alterations in ossification. Collins and Williams (1971) found occasional, dose-related, fused ribs in the offspring of hamsters fed 100, 60, and 40 mg/kg/day during GD6 and 10, and Schwetz et al (1971) determined a decrease in fetal weight and delayed ossification of bone, lumbar ribs, and wavy ribs in rats born to dams dosed with 87.5 mg/kg/day during GD6–15.

The toxicity of 2,4-D and other related compounds is attributed to the free acid form of the chemicals (Munro et al. 1992) and may be mediated by effects associated with the plasma membrane, interference in cellular metabolic pathways involving acetyl coenzyme A, or uncoupling of oxidative phosphorylation (Bradberry et al. 2000). Their herbicide action is mediated through plant hormone pathways, but to the best of our knowledge no solid evidence has been presented to date to support endocrine-like activity of these compounds in mammals or other species. Although the high dose of 2,4-D used in this study (100 mg/kg/day) compares to the doses used previously by other researchers, we did not observe overt toxicity in the dams or in the newborns either at that dose or at any of the lower herbicide mixture dose levels, indicating that there may be inter- or intraspecies differences in

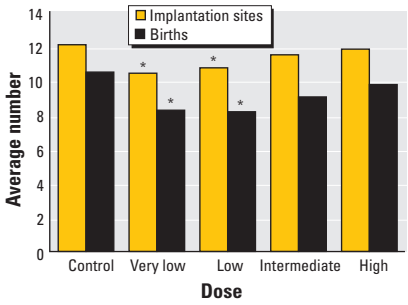

Figure 2. Number of implantation sites and live-born pups after exposure to herbicide mixture.
*$p < 0.05$, ANOVA, Bonferroni.

susceptibility to toxicity. Another possibility is that the interaction between 2,4-D, mecoprop, and dicamba leads to effects different from those of 2,4-D alone or that the inactive ingredients present in the commercial formulation have effects of their own that are more important than those of the active ingredients.

As in any commercial product that requires the delivery of an active ingredient, herbicide formulations include components that ensure the stability of the product during storage, dilution, and application and that maximize the effectiveness of the active ingredient. A typical aqueous herbicide concentrate, such as the one used in this study, is a water-based preparation that includes a water-soluble form of the active ingredient(s), a wetting agent or surfactant, and stability adjuvants such as buffers and preservatives (Tominack 2000). In 1997, the U.S. EPA issued Pesticide Regulation Notice 97-6, which encourages the use of the words "other ingredients" to describe any ingredient in a pesticide product that is not intended to affect a target pest (U.S. EPA 1998).

Oakes and Pollak (1999) studied the effects of the herbicide Tordon 75D and its components 2,4-D and picloram on the oxidative functions of mitochondria and showed that inhibition of oxidation was caused solely by the surfactant present in the formulation. Concentrations of 2,4-D and picloram 136 times higher than those found in the commercial formulation were required to inhibit mitochondria when the surfactant was removed from the experiment. Similarly, Lin and Garry (2000) showed that the commercial-grade herbicides 2,4-D, LV4, and 2,4-D amine induced cell proliferation when added to the medium of a culture of MCF-7 breast cancer cells. The reagent-grade 2,4-D failed to induce cell proliferation over the same concentration range, suggesting that other components of the formulations could have biologic activity.

Although apparently influenced by season, a striking decrease in litter size, approximately 20%, was observed after herbicide administration. This observation is not surprising because fetal death is, in fact, a manifestation of developmental toxicity (Wilson 1973); rather, what is interesting about this observation is the nonlinearity observed in the response, which seems to follow an inverted or U-shaped dose–response pattern. This pattern has also been observed in immune suppression responses to the carbamate aldicarb (Olson et al. 1987). The inverted or U-shaped dose–response model, also known as hormesis, has been reviewed by Calabrese and Baldwin (1999, 2001). The model has been used to describe observations in a wide range of organisms (including bacteria and humans) and chemicals ranging from relatively safe agents to highly toxic compounds (e.g., see Figure 2 in Calabrese and Baldwin 1999). Wilson (1973) stated that "manifestations of deviant development increase in frequency and degree as dosage increases from the no-effect to the totally lethal level" (p. 30), indicating that the expected—and perhaps obvious—dose–litter-size relationship should have been linear.

Hormesis, as defined by Calabrese and Baldwin (2001), is an adaptive response to low levels of stress or damage resulting in improved fitness for some physiologic systems for a finite period. If our observation had been a true hormetic response, we would have observed an increase in litter size at the lower doses and not a decrease. Hormetic responses vary from being enhanced at low doses to being decreased (due to toxicity) at higher doses. Thus, the data we present here do not seem to comply with any of the currently accepted models for dose–response relationships.

A similar nonlinear response was described by vom Saal et al. (1997), who looked at the effects of estradiol on mouse development and found that males exposed to the hormone had enlarged prostates at the lower dose, while the higher dose induced a decrease in prostate weight. vom Saal et al. (1995) also described an inverted U-shaped response between maternal dose of diethylstilbestrol and territorial behavior in male offspring. During our experiments conducted in the fall, we may have had a linear response in which a more pronounced decrease in litter size was produced with higher doses. Unfortunately, we do not have data for the very low dose level administered in the fall. Neither do we have data for number of implantation sites and resorptions, so we cannot conclude that there was a linear response.

Extreme care was taken in our procedures to minimize interexperimental variation so that the animal supplier, the age of the animals at the time of mating, the source of animal food and water, the room temperature and humidity, and the length of the light–dark cycle were always the same in all experiments. We are thus confident that differences observed in response between our experiments may indeed be due to seasonal influences on animal susceptibility. In fact, seasonal variation in the release of reproductive hormones has been reported in experimental animals (Christian 2001).

What is important from these data is the decrease in the number of embryos implanted at the very low and low doses. This observation implies either that preimplantation blastocysts are being negatively affected or that the process of implantation is altered, or that a combination of both mechanisms is occurring. In rodents, uterine receptivity to embryos is modulated by ovarian estrogen and progesterone (Psychoyos 1995). Leutinizing hormone controls progesterone release, and estrogen not only prepares the uterine endometrium but also activates blastocysts for implantation (Yoshinaga 1995). Additionally, copulation in rodents produces surges of prolactin from the pituitary gland, which stimulate the production of uterotrophic progesterone (Cross and Rossant 2001). It is tempting to propose that some sort of endocrine modulation is mediating the effects of the herbicide mixture on litter size, especially since most of the effects were produced by doses at which other environmental chemicals have been shown to produce similar effects. However, this proposal is speculative at this point.

A higher than normal frequency of human births with central nervous system, urogenital, circulatory/respiratory, or musculoskeletal anomalies in western Minnesota has been linked to the use of 2,4-D and other phenoxyacetic acid-derived herbicides (Garry et al. 1996). The authors found that birth anomalies in human males were more common than in females and that the male/female sex ratio in areas of high chlorophenoxy herbicide use was 2.8 for progeny of appliers compared to 1.5 for progeny of the general population of the same area. Curtis et al. (1999) used

Table 4. Implantation sites and resorptions after *in utero* exposure to a commercial mixture of 2,4-D, mecoprop, and dicamba during pregnancy in different seasons (mean ± SD).

Variables/doses	Fall	Winter	Spring	Summer
Implantation sites				
Control	ND	12.1 ± 1.2 (17)	12.4 ± 1.1 (15)	11.7 ± 1.2 (12)
Very low	ND	10.4 ± 0.8 (18)	ND	10.3 ± 2.6 (12)**
Low	ND	ND	11.8 ± 2.1 (18)	9.4 ± 2.7 (15)*
Intermediate	ND	11.6 ± 2.2 (14)	11.8 ± 1.3 (18)	10.9 ± 1.4 (12)
High	ND	12.1 ± 2.2 (13)	12.1 ± 1.6 (19)	9.9 ± 1.0 (13)
Resorptions				
Control	ND	1.6 ± 1.6 (17)	1.4 ± 1.7 (15)	1.1 ± 1.3 (12)
Very low	ND	1.0 ± 0.5 (18)	ND	2.2 ± 1.3 (12)
Low	ND	ND	2.4 ± 1.6 (18)	1.7 ± 1.4 (15)
Intermediate	ND	2.3 ± 2.3 (14)	2.3 ± 1.8 (18)	1.6 ± 1.5 (12)
High	ND	2.4 ± 2.8 (13)	1.8 ± 1.8 (19)	1.3 ± 1.3 (13)

ND, not determined. Pregnant mice were exposed during organogenesis (GD5–15), except for the spring experiment, when they were exposed during preimplantation and organogenesis (GD0–15);
*$p < 0.05$; **$p < 0.01$, ANCOVA, Bonferroni.

conditional fecundity or the monthly probability of conception conditional on pregnancy being achieved during a particular attempt to study the effect of pesticide exposure on time to pregnancy. In their study, a decrease of 20% or more in conditional fecundity was calculated for women engaged in activities using dicamba, glyphosate, phenoxyherbicides, organophosphates, and thiocarbamates. Although 2,4-D and dicamba reportedly do not produce reproductive toxicity (Stevens and Breckenridge 2001), the epidemiologic evidence just discussed and the results presented here imply that further studies are necessary.

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