Low-Dose Agrochemicals and Lawn-Care Pesticides Induce Developmental Toxicity in Murine Preimplantation Embryos

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Abstract and Introduction

Abstract

Occupational exposures to pesticides may increase parental risk of infertility and adverse pregnancy outcomes such as spontaneous abortion, preterm delivery, and congenital anomalies. Less is known about residential use of pesticides and the risks they pose to reproduction and development. In the present study we evaluate environmentally relevant, low-dose exposures to agrochemicals and lawn-care pesticides for their direct effects on mouse preimplantation embryo development, a period corresponding to the first 5-7 days after human conception. Agents tested were those commonly used in the upper midwestern United States, including six herbicides [atrazine, dicamba, metolachlor, 2,4-dichlorophenoxyacetic acid (2,4-D)], pendimethalin, and mecoprop), three insecticides (chlorpyrifos, terbufos, and permethrin), two fungicides (chlorothalonil and mancozeb), a desiccant (diquat), and a fertilizer (ammonium nitrate). Groups of 20-25 embryos were incubated 96 hr *in vitro* with either individual chemicals or mixtures of chemicals simulating exposures encountered by handling pesticides, inhaling drift, or ingesting contaminated groundwater. Incubating embryos with individual pesticides increased the percentage of apoptosis (cell death) for 11 of 13 chemicals (p </= 0.05) and reduced development to blastocyst and mean cell number per embryo for 3 of 13 agents (p </= 0.05). Mixtures simulating preemergent herbicides, postemergent herbicides, and fungicides increased the percentage of apoptosis in exposed embryos (p </= 0.05). Mixtures simulating groundwater contaminants, insecticide formulation, and lawn-care herbicides reduced development to blastocyst and mean cell number per embryo (p </= 0.05). Our data demonstrate that pesticide-induced injury can occur very early in development, with a variety of agents, and at concentrations assumed to be without adverse health consequences for humans.

Introduction

Recent epidemiologic studies suggest that parents working in areas of high pesticide application are at increased risk for adverse reproductive outcomes such as infertility (Fuortes et al. 1997; Greenlee et al. 2003; Smith et al. 1997), poor fertilization (Tielemans et al. 1999), fetal death (Arbuckle and Sever 1998; Saxena et al. 1983), and congenital anomalies (Bell et al. 2001a; Garry et al. 1996, 2002). Residential pesticide exposures and their effects on reproductive health are less well understood. A few studies suggest that maternal exposures to pesticides used around the home are associated with risk of stillbirth and fetal deaths (Bell et al. 2001b; Pastore et al. 1997; Savitz et al. 1989). Decreased birth weight and length of newborns have been associated with high levels of chlorpyrifos in plasma samples of urban minority women (Perera et al. 2003).

Timing, combinations of agrochemicals, duration of exposure, and dose may play critical roles in pregnancy outcomes. Bell et al. (2001a) reported that maternal pesticide exposures occurring during the third to eighth weeks of pregnancy have the greatest impact on fetal deaths. This temporal association strengthened when the pesticides were applied within 1 mi^2 of the maternal residence. Timing of paternal pesticide exposures may also be important. Arbuckle et al. (1999a) reported that exposures to phenoxy herbicides occurring in fathers 3 months before

conception doubled the risk of early spontaneous abortions in their partners.

Pesticide residues have been identified at concentrations of parts per trillion to parts per million in ovarian follicular fluid (Trapp et al. 1984), seminal plasma (Arbuckle et al. 1999b; Foster 1995), human amniotic fluid (Foster et al. 2000), fetal tissue specimens (Nishimura et al. 1977), and meconium from human neonates (Whyatt and Barr 2001). Korrick et al. (2001) and Longnecker et al. (2001) reported that the risk of preterm birth and spontaneous abortion increased with maternal serum concentrations of dichlorodiphenyldichloroethylene (DDE). Fertilization rates have been negatively correlated with levels of DDE in serum and follicular fluids of women undergoing *in vitro* fertilization (Younglai et al. 2002). Little is known, however, about the direct effects of pesticide contaminants on the conceptus and subsequent development near the time of implantation.

Currently, the two-generation Fertility (Reproductive) Assessment by Continuous Breeding protocol developed by the National Toxicology Program (Research Triangle Park, NC) is an accepted method for characterizing developmental and reproductive toxicants [U.S. Environmental Protection Agency (EPA) 1996]. This protocol can be used to evaluate an extensive list of abnormalities in parental and filial generations. However, it is costly and time-consuming, and it does not evaluate exposure risks encompassed by the preimplantation stage of development. The need for more rapid, comprehensive, and cost-effective tools for screening developmental toxicants has stimulated the search for *in vitro* methods to reduce the backlog of chemical testing critical for adequate risk assessment (National Research Council 2000).

We previously demonstrated the potential of the mouse embryo assay for identifying preimplantation toxicity induced by the estrogenic pesticide *o,p´*-dichlorodiphenyltrichloroethane (*o,p´*-DDT) (Greenlee et al. 1999). Compared with control treatment, incubation of pronuclear embryos with 0.1 µg/mL *o,p´*-DDT significantly reduced embryo development to blastocyst and mean cell number and increased the percentage of cells undergoing apoptotic cell death. Developmental effects were dose responsive. Furthermore, the antiestrogen ICI 182,780 abolished the developmental alterations induced by this toxicant (Greenlee et al. 2000), suggesting that the assay may be useful for characterizing injury mechanisms initiated by environmental pollutants with estrogenic activity. Implementation of the mouse preimplantation embryo assay for risk assessment purposes will require further evaluation with a variety of chemicals at ecologically relevant concentrations.

Toward this objective, we screened agricultural and lawn-care chemicals commonly used in the upper midwestern United States as single agents and as mixtures for their effects on embryo development during the preimplantation period. We hypothesized that the mouse embryo assay would prove reliable, rapid, and cost-effective for evaluating pesticide effects at low-dose concentrations and in combinations potentially encountered before a pregnancy is recognized.

Materials and Methods

Animals

All experiments were reviewed and approved by the Marshfield Clinic Institutional Animal Care and Use Committee. Experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996).

Embryo Collection and Culture

CD-1 female mice 21-26 days of age (Charles River Laboratories, Portage, MI) were superovulated with intraperitoneal injections of 5 IU follicle-stimulating hormone (Gestyl; Professional Compounding Center of America, Inc., Houston, TX) followed by 10 IU human chorionic gonadotropin (hCG; Schein Pharmaceutical, Inc., Florham Park, NJ) 47 hr apart. Females were housed with proven CD-1 male mice.

Embryos were collected from the oviducts of female mice with vaginal plugs 18 hr after hCG injection. Reproductive tracts were placed in 37°C modified Earle's balanced salt solution (EMG) (Scott and Wittingham 1996) containing 0.3% bovine serum albumin (BSA; A3311), 0.5 mM glucose (G6152), 1.0 mM glutamine (G1146), 0.05 mM EDTA (E4884), 21.4 mM lactate (L7900), and 0.33 mM pyruvate (P4562) (all from Sigma Chemical Co., St. Louis, MO) and transported to the laboratory in a portable CO_2 incubator (K Systems, Birkerad, Demark). Pronuclear (one-cell zygote) embryos were teased out of the ampullae, and cumulus masses were removed by a 3- to 5-min incubation in 0.2 mg/mL hyaluronidase (H3506) in EMG plus BSA. Embryos were washed through three 3-mL rinses of EMG plus BSA and two 25-µL rinses of EMG without BSA before transferring 20-25 embryos to 25-µL drops of EMG

without BSA containing 0.1% vol/vol ethanol (negative injury control), EMG without BSA with no ethanol (solvent control), 0.1 µg/mL *o,p´*-DDT (positive injury control), individual pesticides, or pesticide mixtures. Embryos were handled in low light using conditions that minimized pH, osmotic, and temperature fluctuations. Microscope stages were heated.

Agrochemicals and Lawn-Care Pesticides

One-cell, pronuclear embryos were incubated with agricultural and lawn-care chemicals at very low-dose concentrations based on the 1× reference dose (RfD) value for each chemical as reported by Kamrin (1997) and the U.S. EPA (2000a). The RfD is an estimate of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime (U.S. EPA 2000a). It is derived from the highest dose level of a chemical that has no adverse effect in LD_{50} animal studies (based on the dose that is lethal to 50% of study animals) divided by a safety factor, typically 100 (Kamrin 1997). RfD units are expressed as milligrams per kilogram of body weight per day. For our experiments, RfD units were converted to milligrams per milliliter, micrograms per milliliter, or nanograms per milliliter because embryos were incubated 4 days *in vitro* with pesticides and fertilizer diluted in culture medium. Cultures were not replenished with pesticides during the incubation period because paracrine factors synthesized by neighboring embryos are essential for optimal embryo development *in vitro* (Brison and Schultz 1997).

The working concentrations of the controls and agrochemicals and the percent purity of the agents are shown in Table 1. Chemical purity was determined by the manufacturers using gas chromatography, mass spectroscopy, flame ionization, or titration. The agrochemicals and lawn-care pesticides tested were those most commonly used in the upper midwestern United States, including six herbicides [atrazine, dicamba, metolachlor, 2,4-dichlorophenoxyacetic acid (2,4-D), pendimethalin, and mecoprop (MCPP)], three insecticides (chlorpyrifos, terbufos, and permethrin), two fungicides (chlorothalonil and mancozeb), one drying agent (diquat), and one fertilizer (ammonium nitrate). Pesticides and desiccant were purchased from AccuStandard, Inc. (New Haven, CT), and ammonium nitrate was purchased from Sigma Chemical Co. Mixtures were prepared using combinations of agrochemicals to simulate preemergence and postemergence (before and after plants break the surface of the ground) herbicide formulations, a fungicide-desiccant combination, groundwater contaminants, insecticide combination, and lawn-care herbicides.

Stock concentrations of agrochemicals were prepared in 100% ethanol (AAPER Alcohol and Chemical Company, Shelbyville, KY). Working concentrations were prepared by serially diluting 10,000× stock solutions to 1× working solutions in EMG without BSA. Stock and working dilutions of mancozeb and diquat were prepared in tissue culture medium because they were more soluble in water than in organic solvents.

Negative and Positive Injury Control Treatments

Two negative control treatments were prepared by supplementing EMG without BSA culture medium with or without 0.1% vol/vol ethanol. Results of the negative injury controls were compared to determine possible developmental effects of the solvent (ethanol) and to identify pesticide treatment effects. The positive injury control treatment was prepared by supplementing EMG without BSA with 0.1 µg/mL *o,p´*-DDT (AccuStandard, Inc.). We selected this pesticide and dose because the treatment reliably induces developmental injury in preimplantation embryos (Greenlee et al. 1999). Results from the negative and positive injury controls provided measures of intra- and interassay variation.

Pesticide-Induced Developmental Injury

At the end of the 96-hr culture period, embryos incubated in test and control treatments were scored for development to blastocyst (Gerrity 1988), percentage of apoptosis, and mean cell number per embryo (Brison and Schultz 1997; Greenlee et al. 1999). Development to blastocyst was determined by identifying the percentage of embryos at the one- to eight-cell, morula, blastocyst, expanded, and hatched blastocyst stages using a Nikon Diaphot inverted microscope fitted with Hoffman differential contrast optics (Modulation Optics Inc., Greenvale, NY) and magnification of 100×. We used the following formula to calculate the percentage of embryos developing to the blastocyst stage:

Percentage of embryos >/= Blastocyst
= (No. of embryos >/= Blastocyst × 100)
÷ Total no. of embryos in culture drop.

Photomicrographs were taken at a magnification of 200× with a Nikon N2000 camera (Nikon) and Kodak Ektachrome ASA 400 film (Eastman Kodak, Rochester, NY). Digital images presented in Figures 1 and 2 were prepared from scanned photographs.

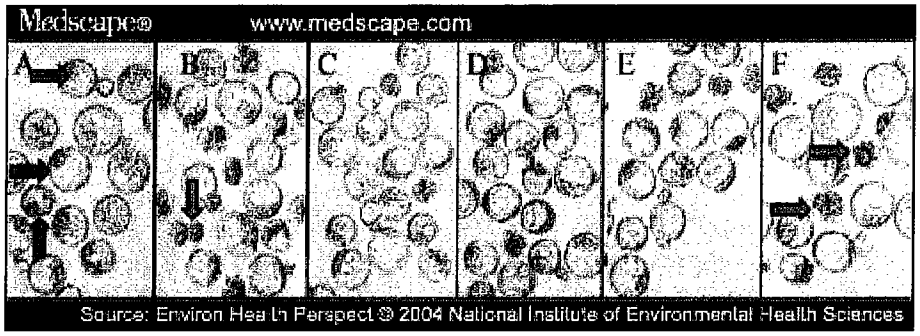

Figure 1. Photomicrograph showing development of embryos cultured 96 hr with (*A*) negative injury control 0.1% (vol/vol) ethanol, (*B*) positive injury control 0.1 µg/mL *o,p´*-DDT, (*C*) 1× RfD dicamba, (*D*) 2,4-D, (*E*) MCPP, or (*F*) a mixture of dicamba, 2,4-D, and MCPP. Magnification, 200×. Red arrows in (*A*) point to embryos at the blastocyst, expanded blastocyst, and hatching blastocyst stages of development. Lavender arrows in (*B*) and (*F*) point to embryos stalled at earlier cleavage stages (two-cell, four- to eight-cell, and morula).

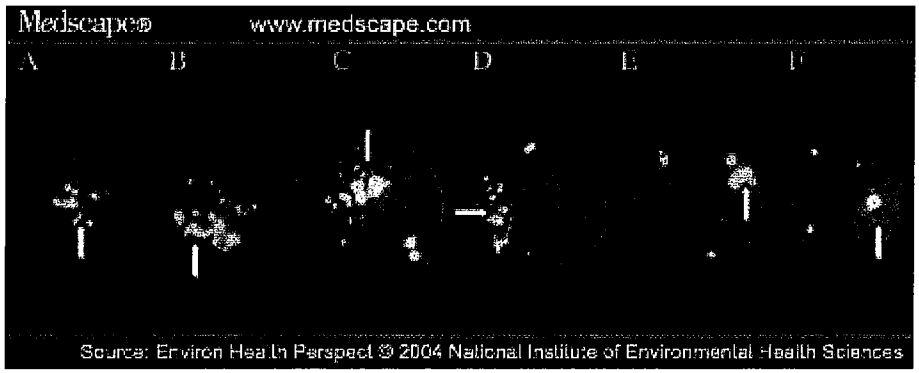

Figure 2. Single embryos from each of the treated groups stained for apoptosis after embryos were cultured 96 hr with (*A*) negative injury control 0.1% (vol/vol) ethanol, (*B*) positive injury control 0.1 µg/mL *o,p´*-DDT, (*C*) 1× RfD dicamba, (*D*) 2,4-D, (*E*) MCPP, or (*F*) a mixture of dicamba, 2,4-D, and MCPP at 1× RfD concentrations. Magnification, 400×. Apoptotic nuclei stain yellow-green and viable cell nuclei stain orange-red. White arrows point to the ICM region of the embryo containing the area of highest apoptotic activity.

We determined the percentage of embryo blastomeres undergoing cell death by apoptosis using the terminal deoxynucleotidyl transferase-mediated 2´-deoxyuridine 5´-triphosphate (dUTP)-biotin nick end-labeling (TUNEL) assay. Blastocysts were fixed overnight in 25-µL droplets of 3.7% paraformaldehyde in phosphate-buffered saline (pH 7.3) (Sigma Chemical Co.) covered with mineral oil (M8410; Sigma Chemical Co.) at 4°C. Fragmented DNA was quantified by labeling the 3´-OH ends of DNA with fluorescein-conjugated dUTP (Apoptosis Detection System; Promega, Inc., Madison, WI). Cell nuclei were counterstained by incubating embryos 20 min in 0.1 mg/mL propidium iodide. Stained embryos were placed in 3-µL drops of Vectashield fluorescence mounting medium (Vector Laboratories, Inc., Burlingame, CA) on numbered glass slides. The test treatments were keyed and examined by a single technician. Cell nuclei were counted at 400× using a Nikon Optiphot-2 microscope fitted with a DAPI/FITC/Rhodamine triple-band pass filter (Nikon). The nuclei of apoptotic cells stained yellow-green, whereas propidium-iodide-stained nuclei of viable cells appeared orange-red. The cell number per blastocyst was determined by combining the counts of orange and green nuclei per embryo. The total number of stained nuclei per embryo was initially counted twice. A third determination was performed if the previous two counts differed by > 5%. The median of the resulting two or three counts was automatically calculated by the database used in the analysis. The percentage of apoptosis was calculated by dividing the number of green nuclei by the total number of nuclei per embryo and multiplying by 100.

Statistical Analysis

Analyses of the primary outcome measures (percentage of embryos developing to blastocyst, the percentage of cells per embryo undergoing apoptosis, and the mean cell number per embryo) were based on analysis of variance (ANOVA) for mixed linear models (SAS Institute Inc. 1997). Experimental replicates, which included negative injury or solvent control, *o,p′*-DDT, and a subset of pesticide and dose combinations (because of limitations on the number of embryos available at a given time) were modeled as a random effect. Batches of embryos served as the unit of analysis, with a mean of 22 embryos per treatment (including control) for the percentage developing to blastocysts and a mean of 13.5 embryos for the percentage of apoptosis and the cell number. Analyses were weighted in proportion to the number of embryos used for a given treatment. This weighted-least-squares approach assumes that observations based on more embryos have lower variability and weights them optimally in the analysis. Each treatment appeared in at least four experiments, all of which included negative and positive controls for reference. Treatment means were computed from the statistical model to incorporate this weighting and to adjust for differences in experimental replicates. As planned by design, each treatment was compared with the control, and the results in this report are deemed statistically significant at the 5% level ($p < 0.05$) without adjustment for multiple comparisons.

Results

Negative Injury and Solvent Control Effects on Embryo Development

We used ethanol to prepare 10,000× stock solutions of 11 of 13 pesticides that were water insoluble. To test for solvent effects on preimplantation development, pronuclear embryos were incubated 96 hr in EMG without BSA supplemented with ($n = 36$) and without ($n = 6$) 0.1% ethanol (negative injury and solvent controls, respectively). This concentration represents the highest possible dose of ethanol in working dilutions of agrochemicals. After the incubation period, significant differences were not detected for the percentage developing to blastocysts (76.4 vs. 76.1%; $p = 0.91$), percentage of apoptosis (10.3 vs. 9.9%; $p = 0.63$), or mean cell number per embryo (111.2 vs. 109.7; $p = 0.67$) for ethanol controls and nonsupplemented controls, respectively.

Developmental Assessment for Embryos Incubated With Pesticides

Embryos were scored for development to blastocyst, mean cell number per embryo, and percentage of apoptosis after 96-hr incubation in controls, individual pesticides, and mixtures at low-dose concentrations based on 1× RfD values. Tables 2-7 provide the weighted means of embryo developmental scores. Compared with the negative injury control treatments, 96-hr incubation of pronuclear embryos with the positive injury control (0.1 µg/mL *o,p′*-DDT) consistently reduced the percentage of development to blastocysts (all $p </= 0.05$) and increased the percentage of blastomeres undergoing apoptosis (all $p </= 0.05$). These findings are similar to those reported in two earlier studies (Greenlee et al. 1999; 2000).

Compared with the negative injury control treatments, incubating embryos with individual agrochemicals significantly increased the percentage of apoptosis for 11 of 13 chemicals tested, including dicamba, pendimethalin, 2,4-D, atrazine, chlorothalonil, mancozeb, diquat, metolachlor, ammonium nitrate, chlorpyrifos, and terbufos (all $p </= 0.05$). One herbicide (atrazine) and two insecticides (chlorpyrifos and turbufos) also reduced embryo development to blastocyst (all $p </= 0.05$). The fertilizer ammonium nitrate reduced mean cell number per embryo ($p </= 0.0005$). A reduction in embryo cell number was the only adverse effect noted ($p </= 0.05$) for the herbicide MCPP.

Mixtures, when compared with negative control treatments, reduced development to blastocyst or increased apoptosis, or had combined effects on blastocyst development and apoptosis. Mixtures formulated to represent preemergent herbicides (dicamba and pendimethalin) and postemergent herbicides (dicamba, 2,4-D, and atrazine) showed a pattern of injury similar to pesticides tested individually; for example, mixtures increased percentage of apoptosis in exposed embryos (all $p </= 0.05$) with no adverse effects on blastocyst development or embryo cell number. In contrast, mixtures formulated to represent groundwater contaminants (atrazine, metolachlor, 2,4-D, and ammonium nitrate), insecticides (chlorpyrifos, terbufos, and permethrin), and lawn-care herbicides (dicamba, 2,4-D, and MCPP) reduced blastocyst development (all $p </= 0.05$). The fungicide mixture (chlorothalonil/ mancozeb/ diquat) reduced development to blastocyst ($p </= 0.05$) and increased the percentage of apoptosis ($p </= 0.005$).

In summary, 12 of 13 individual chemicals and 6 of 6 mixtures at environmentally relevant concentrations induced developmental injury in preimplantation embryos. Only one agent, permethrin, had no measurable effects on developmental outcomes.

Photomicrographs of embryos representative of findings at the end of the culture period are shown in Figures 1 and 2 and correspond to results for control and pesticide treatments presented in Table 7. Figure 1 shows development to blastocyst after incubating groups of 20-25 embryos with the negative (0.1% ethanol) and positive (0.1 µg/mL *o,p'*-DDT) injury controls, individual lawn-care herbicides (dicamba, 2,4-D, or MCPP), or the mixture of lawn-care herbicides (dicamba, 2,4-D, and MCPP). Approximately 70-80% of embryos incubated with the negative injury control (Figure 1A) or with the individual lawn-care herbicides (Figure 1C-E) developed to blastocyst, expanded blastocyst, or hatching blastocyst stages. Embryos at the blastocyst stage of development were characterized as having a thinned zona pellucida, a turgid blastocoele cavity, and a prominent inner cell mass (ICM). Expanded blastocysts showed further thinning of the zonae, with diameters larger than that of the blastocyst stage embryos. Hatching blastocysts exhibited partial protrusion of the embryo through the zona pellucida. Significantly fewer embryos incubated with the positive injury control (*o,p'*-DDT) (Figure 1B) or with the mixture of lawn herbicides (dicamba, 2,4-D, and MCPP) (Figure 1F) progressed to blastocyst (58-65%; all p </= 0.05). Residual embryos in *o,p'*-DDT, the positive injury control, and the herbicide mixture were often stalled at early cleavage stages (e.g., two-, four-, and eight-cell, and morula). Residual embryos in the negative injury control and individual pesticide treatment drops were frequently stalled at later cleavage stages (e.g., morula and preblast).

Photomicrographs shown in Figure 2 illustrate apoptosis results for embryos incubated in negative (Figure 2A) and positive (Figure 1B) injury controls and pesticide treatments (Figure 2C-F) detailed in Figure 1. The highest percentages of apoptosis were observed for embryos incubated with the positive injury treatment 0.1 µg/mL *o,p'*-DDT (Figure 2B) and for embryos incubated with the individual herbicides dicamba, 2,4-D, and MCPP (Figure 2C-E) (all p </= 0.05). The percentage of apoptosis for the herbicide mixture (Figure 2F) was not significantly different from the negative injury control (Figure 2A). Apoptotic nuclei were observed most commonly in the region of the ICM of blastocysts and stained yellow-green with TUNEL reagents.

Discussion

Our data demonstrate that pesticide-induced injury can occur at a very early period of embryo development and at pesticide concentrations assumed to be without adverse health consequences for humans. Embryo injury was noted for single agents and for mixtures at concentrations based on 1× RfD values. The RfD is an estimate of a daily exposure to the human population assumed to be of negligible risk for deleterious effects during a lifetime. The RfD is derived by dividing the no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) dose by uncertainty factors to accommodate limitations in data, variability within humans, and differences in responses of test and target species. The use of the NOAEL/LOAEL has been criticized because of its sensitivity to sample size, high sampling variability from experiment to experiment, and the inability to use all dose-response data (Barnes et al. 1995; U.S. EPA 2000b). Future studies to evaluate risks of adverse exposures may be better served by using benchmark dose modeling because it is more inclusive of dose-response data and better reflects sample size (Castorina and Woodruff 2003).

Our findings may have implications for human reproductive health. Embryos cleaving to blastocyst yet undergoing cellular death at a higher rate could result in embryos composed of fewer cells. Unless repair mechanisms overcome cellular loss, exposures during this period could result in embryonic demise, implantation failures, or alterations in the physiologic processes underlying maternal recognition of pregnancy (Wilson 1973). Findings from animal dosing studies are consistent with these possibilities. Pregnant mice exposed to very low and low doses of an herbicide mixture (2,4-D, MCPP, and dicamba) during the period of preimplantation-organogenesis (gestation days 0-15) resulted in significant reductions in implantation sites and live births (Cavieres et al. 2002). Female mice receiving oral administration of the insecticide lindane either before or immediately after mating increased blastomere lysis and suppressed cell proliferation of two-cell embryos and morulae (Scascitelli and Pacchierotti 2003). Mice receiving subcutaneous injections of the insecticide methoxychlor on days 2-4 of pregnancy yielded embryos exhibiting suppressed blastocyst proliferation, increased percentages of nuclear fragmentation (apoptosis), and micronuclei formation (Amstislavksy et al. 2003). Embryos collected from female mice receiving a single intraperitoneal injection of the insecticide chlorpyrifos on day 0 of pregnancy showed significant increases in micronucleus formation and a dose-dependent reduction in embryo cell numbers (Tian and Yamauchi 2003). Therefore, our findings for *in vitro* exposed embryos closely parallel those observed for embryos collected from the reproductive tracts of mice dosed during the preimplantation period. The relevance of preimplantation embryo injury to pregnancy outcomes needs further clarification. This might be accomplished by transferring *in vitro* exposed embryos to foster mice and monitoring implantation rate, litter size, and pup normalcy at birth.

Agrochemicals and lawn-care pesticides were tested at concentrations ranging between parts per trillion for the insecticide terbufos to parts per billion for the herbicide metolachlor. These concentrations are environmentally relevant and physiologically achievable based on pesticide levels reported for human follicular aspirates (Baukloh et

al. 1985; Jarrell et al. 1993) and for maternal and cord blood samples collected at delivery (Waliszewski et al. 2000). Comparisons between contaminant concentrations in maternal and cord plasma samples suggest a balanced state between mother and fetus with respect to circulating pesticides and metabolites (Whyatt et al. 2003). Similar correlations between maternal serum and follicular fluid contaminant levels have been reported for *in vitro* fertilization patients (Younglai et al. 2002). Just before ovulation, follicles become highly vascularized (Edwards et al. 1980). This increased blood flow may enhance transfer and accumulation of pollutants from serum to follicular fluids (Baukloh et al. 1985).

Adjuvants (paraffinic oils and/or surfactant mixtures) were not included in the test formulations. Adjuvants are typically combined with the active ingredients in commercial formulations to improve the characteristics of penetration, spreading, or longevity in the field (Tominack 2000). Adjuvants alone may have disruptive effects, as demonstrated by growth promotion of human tumor cell lines (Lin and Garry 2000), abnormal endocrine profiles of pesticide/adjuvant applicators (Garry et al. 1999), and cell cycle delays in embryo cleavage (Marc et al. 2002). In the latter study, pesticide toxicity was detected only in combination with a subthreshold concentration of a commercial pesticide formulation containing inert ingredients. In our study, individual agents and mixtures of agrochemicals caused measurable injury without the addition of adjuvants. It will be important to determine if embryo development is further compromised by combining pesticides with other ingredients found in commercial formulations.

Ethanol is a known teratogen. Maternal ingestion of at least 0.5 oz (14 mL) per day during pregnancy has resulted in measurable neurodevelopmental abnormalities in young children (Sood et al. 2001). This dose approximates a daily body burden of 0.01-0.03% ethanol and may vary based on the weight and genetic factors of the woman. Ethanol was used as a solvent for 11 of 13 pesticides. The 0.1% ethanol control represents the highest possible concentration of solvent. No differences in developmental parameters were measured for embryos incubated 96 hr in medium with and without ethanol (all $p > 0.63$). However, it is possible that ethanol supplementation at this concentration may have latent deleterious effects. Additional studies are needed to fully address this question.

Exposure to single agents and certain mixtures elevated percent cell death without affecting development to blastocyst. Other treatments stalled development to blastocyst without increasing apoptotic cellular death. For example, dicamba alone or combined with pendimethalin or 2,4-D and atrazine induced significant levels of apoptosis. However, dicamba combined with 2,4-D and MCPP significantly reduced development to blastocyst without increasing rates of cell death (Table 2, Table 3, and Table 7). One explanation may be that early cleavage-stage embryos were not competent to initiate apoptosis. However, this is unlikely, as the requisite molecular components for the apoptotic cascade are available in the blastomeres of embryos at all stages of development (Weil et al. 1996). Another possibility to explain differing injury profiles may be the combined effects of three compounds rather than a single agent. It is believed that embryos must first differentiate into distinct embryonic regions, the ICM and the trophectoderm (TE), before apoptosis is engaged. The temporal significance of apoptosis may be to rid TE cells from the rapidly growing ICM (Pierce et al. 1989). In support of this possibility, Hardy (1999) and Brison and Schultz (1997) noted that most apoptotic activity was confined to the ICM region of blastocyst embryos. We also localized apoptosis primarily in the region of the blastocyst ICM (Figure 2). Therefore, embryos may need to cleave normally to blastocyst to demonstrate an increased vulnerability to low-dose contaminants. More substantial injuries, causing embryos to stall differentiation to ICM and TE, would result in rates of apoptosis similar to the negative control treatment.

Agrochemicals and lawn-care pesticides chosen for testing are those still commonly used in the upper midwestern United States. Mixture formulations were based on possible exposures routes (e.g., ingesting contaminated groundwater; mixing and handling pesticides; inhaling pesticide drift). Compounds could also be screened based on common mechanisms of pesticide action. The embryo model is well suited for accommodating both approaches.

Conclusions

The mouse preimplantation embryo assay appeared sensitive and reliable for assessing early developmental injury due to agrochemical exposures at concentrations below which health effects are thought to occur. *In vitro* exposure of murine preimplantation embryos to the negative and positive injury control treatments provided reproducible comparisons for pesticide treatment effects on developmental outcomes (blastocyst development, embryo cell number, and percentage of apoptosis). Results of this study may assist with modeling risk of agrochemical exposures coinciding with events of early pregnancy. However, additional efforts are needed to validate the assay for purposes of human risk assessment and to determine the relevance of *in vitro* exposures to pregnancy outcomes.

Tables

Table 1. Working concentrations and purity of agrochemicals and lawn-care pesticides tested individually or as mixtures for their effects on preimplantation embryo development

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Chemical	Working concentration[a] (µg/mL)	Percent purity[b]
Negative injury control		
Ethanol	0.1% vol/vol	≥ 99.5
Positive injury control		
o,p'-DDT	0.1	≥ 97.4
Preemergence herbicides		
Dicamba	0.030	≥ 99.1
Pendimethalin	0.040	100
Dicamba/pendimethalin	0.03/0.04	≥ 99.1/100
Postemergence herbicides		
Dicamba	0.030	≥ 99.1
2,4-D	0.010	≥ 99.1
Atrazine	0.035	100
Dicamba/2,4-D/atrazine	0.03/0.01/0.035	≥ 99.1/≥ 99.1/100
Fungicides/dessicant		
Chlorothalonil	0.015	≥ 99.8
Mancozeb	0.003	100
Diquat	0.0022	99
Chlorothalonil/mancozeb/diquat	0.015/0.003/0.0022	≥ 99.8/100/99
Groundwater contaminants		
Atrazine	0.035	100
Metolachlor	0.100	≥ 97.5
2,4-D	0.010	≥ 99.1
Ammonium nitrate	1.000	99.5
Atrazine/metolachlor/2,4-D/ammonium nitrate	0.035/ 0.1/0.01/1.0	100/≥ 97.5/≥ 99.1/99.5
Insecticides		
Chlorpyrifos	0.003	100
Terbufos	0.0001	99.4
Permethrin	0.050	≥ 96.9
Chlorpyrifos/terbufos/permethrin	0.003/0.0001/0.05	100/99.4/≥ 96.9
Lawn care herbicides		
Dicamba	0.030	≥ 99.1
2,4-D	0.010	≥ 99.1
MCPP	0.0005	98.7
Dicamba/ 2,4-D/MCPP	0.03/0.01/0.0005	≥ 99.1/≥ 99.1/98.7

[a]Working dilutions were based on 1× RfD (mg/kg/day) as provided by Kamrin (1997) and the U.S. EPA (2000a). [b]Purity was determined by the manufacturers using gas chromatography, mass spectroscopy, flame ionization, or titration.

Table 2. Preemergence herbicides dicamba and pendimethalin tested individually and as a mixture at lowdose concentrations for effects on murine preimplantation embryo development

Treatment[a]	Percentage developing blastocysts[b]	Percentage of apoptosis[b]	Mean cell no./embryo[b]
0.1% ethanol (negative injury control)	72.20 ± 3.51	9.99 ± 0.52	105.44 ± 2.76
0.1 µg/mL *o,p'*-DDT (positive injury control)	55.64 ± 3.50*	13.05 ± 0.51***	103.47 ± 2.72
Dicamba	80.24 ± 5.10	12.39 ± 0.63*	102.46 ± 3.27
Pendimethalin	76.70 ± 5.16	12.47 ± 0.64*	99.60 ± 3.36
Dicamba/pendimethalin	62.91 ± 5.04	12.01 ± 0.67*	101.97 ± 3.50

[a]Groups of 20–25 embryos were exposed for 96 hr to working dilutions of pesticides at low-dose concentrations based on 1× RfD values shown in Table 1. [b]Values are weighted means calculated from the results of at least four experiments ± SEs. *$p \le 0.05$ and ***$p \le 0.0005$ calculated by ANOVA against comparisons with the negative injury control (0.1% ethanol).

Table 3. Postemergence herbicides dicamba, 2,4-D, and atrazine tested individually and as a mixture at low-dose concentrations for effects on murine preimplantation embryo development

Treatment[a]	Percentage developing blastocysts[b]	Percentage of apoptosis[b]	Mean cell no./embryo[b]
0.1% ethanol (negative injury control)	74.83 ± 2.19	10.27 ± 0.47	109.22 ± 2.59
0.1 µg/mL *o,p'*-DDT (positive injury control)	57.79 ± 2.30***	13.04 ± 0.48***	101.24 ± 2.63*
Dicamba	80.24 ± 4.04	12.70 ± 0.70**	102.55 ± 3.56
2,4-D	77.35 ± 3.97	12.92 ± 0.70**	102.29 ± 3.62
Atrazine	67.06 ± 3.97	13.58 ± 0.76***	105.28 ± 3.88
Dicamba/2,4-D/atrazine	71.94 ± 4.05	12.28 ± 0.76*	102.34 ± 3.98

[a]Groups of 20–25 embryos were exposed for 96 hr to working dilutions of pesticides at low-dose concentrations based on 1× RfD values shown in Table 1. [b]Values are weighted means calculated from the results of at least four experiments ± SEs. *$p \le 0.05$, **$p \le 0.005$, and ***$p \le 0.0005$ calculated by ANOVA against comparisons with the negative injury control (0.1% ethanol).

Table 4. Fungicides and desiccant chlorothalonil, mancozeb, and diquat tested individually and as a mixture at low-dose concentrations for effects on murine preimplantation embryo development

Treatment[a]	Percentage developing blastocysts[b]	Percentage of apoptosis[b]	Mean cell no./embryo[b]
(±) 0.1% ethanol (combined negative controls)[c]	77.19 ± 1.36	10.26 ± 0.32	110.20 ± 1.58
0.1 µg/mL *o,p'*-DDT (positive injury control)	57.62 ± 1.39***	12.84 ± 0.32***	105.32 ± 1.61**
Chlorothalonil	71.87 ± 3.09	12.54 ± 0.67**	108.17 ± 3.14
Mancozeb[d]	73.48 ± 3.73	13.62 ± 0.79***	103.23 ± 3.67
Diquat[d]	76.04 ± 3.72	14.12 ± 0.82***	106.68 ± 3.79
Chlorothalonil/mancozeb/diquat	68.36 ± 3.76*	13.05 ± 0.84**	109.88 ± 3.87

[a]Groups of 20–25 embryos were exposed 96 hr to working dilutions of pesticides at low-dose concentrations based on 1× RfD values shown in Table 1. [b]Values are weighted means calculated from the results of at least four experiments ± SEs. [c]The negative injury control results are data combined from EMG without BSA supplemented with ($n = 36$) or without ($n = 6$) 0.1% ethanol. [d]Mancozeb and diquat were not soluble in ethanol; pesticide stock and working dilutions were prepared in EMG without BSA. *$p \le 0.05$, **$p \le 0.005$, and ***$p \le 0.0005$ calculated by ANOVA against results of the combined negative control treatments.

Table 5. Groundwater contaminants atrazine, ammonium nitrate, 2,4-D, and metolachlor tested individually and as a mixture at low-dose concentrations for effects on murine preimplantation embryo development

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Treatment[a]	Percentage developing blastocysts[b]	Percentage of apoptosis[b]	Mean cell no./embryo[b]
0.1% ethanol (negative injury control)	78.10 ± 2.61	10.53 ± 0.51	110.73 ± 2.75
0.1 µg/mL *o,p'*-DDT (positive injury control)	59.33 ± 2.69***	13.05 ± 0.53**	103.94 ± 2.79**
Atrazine	67.61 ± 4.47*	13.52 ± 0.96**	107.23 ± 3.94
Metolachlor	73.51 ± 4.46	12.53 ± 0.89*	100.81 ± 3.78*
2,4-D	77.03 ± 4.47	13.00 ± 0.87*	104.32 ± 3.72
Ammonium nitrate	68.92 ± 4.44	13.29 ± 0.96*	96.98 ± 3.92***
Atrazine/metolachlor/2,4-D/ammonium nitrate	62.98 ± 4.51*	11.72 ± 0.97	106.36 ± 3.98

[a]Groups of 20–25 embryos were exposed 96 hr to working dilutions of pesticides at low-dose concentrations based on 1x RfD values shown in Table 1. [b]Values are weighted means calculated from the results of at least four experiments ± SEs. *$p \leq 0.05$, **$p \leq 0.005$, and ***$p \leq 0.0005$ calculated by ANOVA against comparisons with the negative injury control (0.1% ethanol).

Table 6. Insecticides chlorpyrifos, terbufos, and permethrin tested individually and as a mixture for effects at low-dose concentrations on murine preimplantation embryo development

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Treatment[a]	Percentage developing blastocysts[b]	Percentage of apoptosis[b]	Mean cell no./embryo[b]
0.1% ethanol (negative injury control)	81.47 ± 2.51	10.63 ± 0.61	111.69 ± 2.89
0.1 µg/mL *o,p'*-DDT (positive injury control)	62.35 ± 2.58***	13.20 ± 0.63***	106.09 ± 2.99
Chlorpyrifos	71.76 ± 4.09*	13.31 ± 0.92*	103.08 ± 4.51
Terbufos	71.43 ± 4.05*	12.87 ± 0.92*	111.41 ± 4.50
Permethrin	74.72 ± 4.09	11.71 ± 0.95	107.47 ± 4.66
Chlorpyrifos/terbufos/permethrin	66.86 ± 3.73**	11.96 ± 0.85	104.39 ± 4.13

[a]Groups of 20–25 embryos were exposed 96 hr to working dilutions of pesticides at low-dose concentrations based on 1x RfD values shown in Table 1. [b]Values are weighted means calculated from the results of four to five experiments ± SEs. *$p \leq 0.05$, **$p \leq 0.005$, and ***$p \leq 0.0005$ calculated by ANOVA against comparisons with the negative injury control (0.1% ethanol).

Table 7. Lawn care herbicides dicamba, 2,4-D, and MCPP tested individually and as a mixture at low-dose concentrations for effects on murine preimplantation embryo development

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Treatment[a]	Percentage developing blastocysts[b]	Percentage of apoptosis[b]	Mean cell no./embryo[b]
0.1% ethanol (negative injury control)	74.12 ± 2.00	10.10 ± 0.39	109.77 ± 2.27
0.1 µg/mL *o,p'*-DDT (positive injury control)	58.02 ± 2.08***	12.63 ± 0.40***	102.27 ± 2.32*
Dicamba	80.33 ± 3.77	12.51 ± 0.64**	103.00 ± 3.65
2,4-D	76.52 ± 3.71	12.87 ± 0.64***	103.31 ± 3.65
MCPP	69.83 ± 3.77	11.40 ± 0.68	100.25 ± 3.85*
Dicamba/2,4-D/MCPP	64.68 ± 3.71*	11.13 ± 0.69	99.50 ± 3.92*

[a]Groups of 20–25 embryos were exposed 96 hr to working dilutions of pesticides at low-dose concentrations based on 1× RfD values shown in Table 1. [b]Values are weighted means calculated from the results of at least four experiments ± SEs. *$p \le 0.05$, **$p \le 0.005$, and ***$p \le 0.0005$ calculated by ANOVA against comparisons with the negative injury control (0.1% ethanol).

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