ABSTRACT

Intraspecies variability is an important component in evaluating public health risks. In developmental toxicity studies, such variation may occur among litters within a treatment group. This study examined the degree of similarity across three breedings of female mice (CD-1) with respect to their responses to two known developmental toxicants. Each female was mated to the same male (CD-1) and exposed to the same dosing regimen during each pregnancy. Litters were evaluated for postnatal viability and growth. The known murine teratogens dinocap and all-trans retinoic acid were chosen because of the existence of adequate historical data on these compounds. Two doses of each chemical were chosen to produce marginal or severe developmental toxicity. Five treatment groups (22-29 animals per group) were dosed by gastric intubation as follows: control (corn oil, gestation days 10 through 13); dinocap (20 or 40 mg/kg/day in corn oil, gestation days 10 through 13); and all-trans retinoic acid (10 or 20 mg/kg/day in corn oil, gestation days 11 through 13). Litters were examined on postnatal days 1 and 3 for viability, evidence of developmental toxicity, and growth (body weight). Gross abnormalities observed included abdominal ballooning in the high dose litters and limb defects in the all-trans retinoic acid treated animals. Intergroup differences were assessed using the multiple t-test of least square means. A rank test of independence was performed to assess whether or not a given dam's response was consistent across litters and intraclass correlation coefficients were calculated to assess between versus within dam variability. These analyses indicated that maternal variability contributed to interlitter difference in untreated CD-1 mice, in many cases up to 40 percent. Treatment with either of two known development toxicants, however, decreased the role that maternal variability played with respect to interlitter differences, frequently dropping by greater than 50 percent.

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INTRODUCTION

Historically, in the regulation of industrial or agricultural chemicals by government agencies, uncertainty factors have been applied to exposure or dose levels observed in epidemiological or toxicological studies in order to estimate an acceptable environmental or industrial level of exposure for humans. Differences in toxicological response related to intraspecies variability are generally accounted for by such uncertainty factors.

Most commonly, data derived from laboratory animals or occupational studies of "healthy" workers are used to calculate an acceptable exposure level for the general human population including sensitive individuals. More specifically, for noncancer health endpoints, an evaluation of a chemical's toxicity for regulatory purposes has traditionally involved the identification of a lowest-observed-adverse-effect level (LOAEL) or no-observed-adverse-effect level (NOAEL). The LOAEL or NOAEL is converted to a human equivalent dose followed by division of this dose by uncertainty factors. Uncertainty factors (generally factors of 10 for each applicable source of uncertainty) are generally used to account for (1) interspecies variability; (2) intraspecies variability; (3) use of a LOAEL when a NOAEL is not available; and (4) use of health data associated with subchronic rather than chronic exposures when deriving an acceptable level for long-term exposure periods. This procedure is commonly referred to as the derivation of a reference dose (RfD). A discussion of the history and limited experimental evidence supporting the uncertainty factors has been previously presented (Dourson and Stara,

1983). A discussion of the RfD development and verification process is available elsewhere (Barnes and Dourson, 1989; U.S. EPA, 1987).

Despite the use of an uncertainty factor of ten to account for intraspecies variability, the true significance of this variability in response to a toxicant is often unknown. Generally, studies are designed with sufficient numbers of animals so that the agent-induced effects may be identified regardless of normal genetic variation. Studies conducted using multiple strains which would allow for exploring the diversity of a species are relatively uncommon (Festing, 1990).

In many toxicological studies, at intermediate dose levels, a wide variety of responses may be observed in different animals. Frequently, a small number of animals within a treatment group may appear to be either sensitive or resistant to a dose level, though this observation may not be statistically significant. In developmental toxicity studies, this observation may be seen as: (1) some litters appearing to be unaffected by a given chemical/dose, while others are severely affected, and (2) litters containing substantial within-litter variation in response, with resorbed or dead conceptuses, malformed fetuses, and normal fetuses in the same litter. Dams evaluated in developmental toxicity studies are commonly sacrificed prior to or soon after delivery of their first litters, therefore, further study of those animals producing highly sensitive, resistant, or variable litters is not generally possible.

The phenomenon of intraspecies variability may have a strong genetic component resulting from a difference in a dam's ability to distribute or metabolize a teratogen, a variation in the uterine environment, or a difference in a contributing factor

transmitted through the egg but not the sperm cytoplasm (Biddle, 1981; Biddle and Fraser, 1977). In addition to maternal genetic variability, other factors that may account for intraspecies differences include: experimental error and/or variation (e.g., in dosing volume), paternal genetic variability, the amount of food in the stomach of animals dosed orally, differences in the health status of dams which may not be apparent to the investigator, and kinetic differences based upon variations in the size of a litter or position within the uterus.

A number of studies have attempted to elucidate the precise roles of the maternal and/or fetal genomes in in utero growth. These studies have generally utilized egg transplant techniques or treatments in inbred strains (Biddle and Fraser, 1977; Inouye and Kajiwara, 1990; Kalter, 1979; Seller et.al.,1983; Hansen and Hodes, 1983). However, no study has directly dealt with the role of individual maternal variation in developmental toxic responses.

An outbred strain of laboratory animals was chosen to study intraspecies variation as these animals provide the investigator with a population having genetic heterogeneity and strong reproductive characteristics. Conversely, an inbred strain composed of virtually genetically equivalent individuals provides only a narrow spectrum of the genetic makeup of a species, generally less reproductive vigor, and, reportedly, increased sensitivity to phenotypic variance caused by environmental factors (Gill, 1980).

A modification of an in vivo tertology screen developed by Chernoff and Kavlock provided a mechanism for studying multiple litters produced by the same parents and exposed in utero to the same treatment regime. More specifically, the

Chernoff-Kavlock screening assay involves exposing pregnant animals to a minimally toxic dose during a portion of the period of major organogenesis. The dams are allowed to give birth and litter size and pup weight are examined on postnatal days 1 and 3 (Chernoff and Kavlock, 1982). Results of previous studies using this protocol have shown that most prenatal effects can be observed postnatally as reduced viability and/or impaired growth (Chernoff and Kavlock, 1982; Gray and Kavlock, 1984; Kavlock, et al., 1987). Specific modifications to this study design needed to evaluate the role of maternal variation in developmental toxicity include: expanding the protocol in order to evaluate multiple litters of the same parents; selecting dose levels that produce little or no maternal toxicity since it was desired to maintain the health of the females across multiple breedings and because developmental effects observed at nonmaternally toxic levels are of most concern; and dosing dams at more than one level per chemical.

Two known murine teratogens, dinocap and all-trans retinoic acid, were chosen for the study based on results of pilot studies and because of the existence of adequate historical data on these compounds. A brief summary of each chemical is presented below.

Dinocap is a complex mixture used as a fungicide in the control of powdery mildew and also as a miticide (Rohm and Haas Co., 1961). Technical-grade dinocap reportedly contains 74% 2,4- and 2,6- dinitrooctylphenylcrotonates, 6% mixed nitrooctylphenols, and 0.54 - 0.86% mononitrooctyl- phenols, with nonvolatile materials accounting for the remaining 6- 13% (Kurtz et.al., 1970). The technical-grade dinocap, administered orally in corn oil, has been shown to be a development toxicant in the CD-

1 mouse at levels that are not maternotoxic. Adverse effects seen in the offspring include cleft palate, otolith defects, reduced body weight, abdominal "ballooning", poor postnatal survival, and behavioral torticollis (Gray et. al., 1986; Rogers et. al., 1986; Rogers et. al., 1987a). The standard teratogenicity study underestimates the toxic effects in the CD-1 mouse. Specifically, behavioral effects that are not observed at birth may be seen at weaning (Gray et. al, 1988). The active component(s) within the technical-grade dinocap responsible for the developmental toxicity is (are) unknown since neither of the two major fungicidal ingredients (2,4-dinitro-6-(1-methylheptyl)phenyl crotonate and 2,6-dinitro-4-(1-methylheptyl)phenyl crotonate produce developmental toxicity in purified form (Rogers et. al., 1987b).

All-trans retinoic acid is a naturally occurring form of vitamin A. This vitamin can be metabolized to retinoic acid which has been proposed to be a morphogen in limb development (Thaller and Eichele, 1987; Satre and Kochhar, 1988; Maden, 1985; Thaller and Eichele, 1988). Excess vitamin A or retinoic acid has been found to be teratogenic in many animal species, producing a wide range of malformations including cleft palate and limb defects (Geelen, J.A.G., 1979; Kochhar et. al., 1984; Birnbaum et.al., 1989). In the ICR mouse, the period of peak susceptibility for limb development is day 11.5 of gestation and that for palatal clefts, day 12.0 (sperm plug = day 1) (Kochhar et. al., 1984).

In standard developmental toxicity studies, the pregnant animal is killed before paturition, and each animal is bred only once. Is the variability among litters due to chance, or are the responses of the individual dams intrinsic? This study examined the

degree of similarity across breedings of a given female in terms of its response to a known developmental toxicant. The hypothesis, that maternal variability contributes significantly to inter-litter difference, was examined in a group of multiply bred animals with constant dosage. The study design followed an in vivo screening procedure that relies on postnatal viability and growth as measures of developmental toxicity (Chernoff and Kavlock, 1982).

MATERIALS AND METHODS

Animals

Females, approximately 60 days old, and males, approximately 90 days old, of the CD-1 strain were obtained from Charles River Laboratories (Raleigh, NC). Animals were housed in solid bottom cages with wood shavings bedding and fed Wayne Lab Blox and tap water ad libitium. The animal rooms were maintained at 20 to 24° C and 40 to 50 percent relative humidity. The mice were kept on a 10 hours light:14 hours dark photoperiod and were housed individually except during breeding periods.

Chemicals

Two pilot studies were undertaken to determine the chemicals and dosing regimes to be used in this study. Five chemicals (nitrofen, dinocap, valproic acid, all-trans retinoic acid, and 2,4,5 trichlorophenoxyacetic acid (2,4,5-T)) were assessed in the first pilot study. All of these chemicals are known developmental toxicants in the mouse. Based upon the results of this pilot study, three chemicals (nitrofen, dinocap, and all-trans retinoic acid) were further evaluated in a second pilot study. Two chemicals (dinocap and all-trans retinoic acid) were chosen for the definitive study. Treatment

levels were selected to produce minimal or no maternal toxicity. The two dosage levels selected for each chemical were chosen to produce developmental effects in approximately 25 to 35 percent of the low dose litters and effects in approximately 50 to 75 percent of the high dose litters. The results of the two pilot studies are presented in the Appendix.

Experimental Design

Each male was initially mated with five females chosen randomly. The mating period lasted 10 days (two Sunday night through Thursday night periods). Females were checked the morning after mating for evidence of a sperm plug (gestation day 0). As each female successfully mated, it was removed from the male's cage and assigned randomly to one of five treatment groups. Only one female mated to a specific male was assigned to a given treatment group. Females that did not successfully mate during the 10 day breeding period were removed from the study.

The five treatment groups were as follows: (1) control (corn oil); (2) 20 mg/kg/day dinocap; (3) 40 mg/kg/day dinocap; (4) 10 mg/kg/day all-trans retinoic acid; and (5) 20 mg/kg/day all-trans retinoic acid. Control and dinocap-treated dams were dosed on days 10 - 13, and retinoic acid-treated dams were treated on days 11 - 13. The dose levels were selected based upon the two pilot studies presented in the Appendix. The compounds were administered by gastric intubation using corn oil as the vehicle. Dosing solutions were adjusted for chemical purity. The females were weighed on gestation days 0, 8, 17 and throughout dosing. Dosing volumes were adjusted daily for each dam's individual body weight and ranged from 0.2 to 0.6 milliliters per day.

Throughout gestation, the dams were observed for signs of toxicity. The females were allowed to deliver naturally and the litters were examined on postnatal days 1 and 3 for viability, evidence of developmental toxicity, and growth (body weight). Following litter observations on postnatal day 3, all surviving offspring were removed from the dam and euthanized. Dams that had not delivered litters by postnatal day 3 and that had shown little or no weight gain during the gestational period were assumed to be nonpregnant and were removed from the study.

Approximately one week following the last litter's postnatal day 3, each female that had successfully delivered a first litter was remated to its original mate. The protocol described above was followed a second time. Similarily, dams that successfully delivered a second litter were remated to the same males for a third time. Only dams that successfully delivered three litters were retained in the study. Treatment groups ultimately contained 22 to 29 animals. The length of time from the first mating through delivery of the third litter was approximately 100 days.

Exposure to dinocap has been shown to increase absolute and relative liver weight (Rogers et.al., 1986). Therefore, following postnatal day 3 of the third litters, all control and dinocap females were killed by asphyxiation with carbon monoxide and whole body and liver weights were recorded.

Statistical Analyses

To evaluate intergroup differences, the multiple t-test of least squares means using the General Linear Models procedure of the Statistical Analysis System was executed (SAS, 1985). The litter was used as the statistical unit for all comparisons;

therefore, the values presented in Tables I - IX represent least squares means of litter values $\pm$ standard error. Values that were statistically different from controls at $p \leq 0.05$ and $p \leq 0.01$ were noted.

To assess whether or not a response was consistent rather than random across breedings, a rank test of independence was performed (Lehmann, 1975). The correlation of the ranking of variables between two litters (litters 1 and 2; litters 2 and 3; and litters 1 and 3) were determined. For each litter and variable (e.g., the number of live neonates/litter on postnatal day 1), dams were ranked within each treatment group. Thus, the dam with the largest number of live neonates/litter on postnatal day 1 would be given a rank of 1, the dam with the second largest number of live neonates/litter on postnatal day 1 would receive a rank of 2, etc. Rank ties were given equivalent scores. A dam's rank for a specific variable could then be compared across litters. The null hypothesis (H_o) tested was that the dams will respond randomly across multiple breeds and, thus, a dam's rank for a given variable will change significantly from litter to litter. Where the H_o could be rejected at the $p \leq 0.05$ level, the dams/litters were determined to be responding consistently from litter to litter.

Intraclass correlation coefficients were calculated to assess the between versus within dam variability; that is, the significance of the ratio of interdam variability to interdam plus intradam variability. A ratio of 0.50 would indicate that interdam and intradam variability contributes equally to interlitter differences. Confidence intervals were calculated for the intraclass correlation coefficients and values that were

significantly different from control animals were identified (Donner and Wells, 1986; Swiger et.al., 1964).

RESULTS AND DISCUSSION

The results of exposing pregnant CD-1 mice to the the five treatment regimens are presented in Tables I through XV and Figure I. Summaries of maternal and neonatal toxicity are presented below.

Maternal Effects

As identified in the previous section, only dams that successfully delivered three litters remained in the study for statistical analyses. Twenty-two to 29 animals per treatment group successfully delivered three litters. Of the dams not considered in the statistical analyses, less than two percent died during any part of the study, therefore, treatment-related maternal mortality was considered unimportant.

Evidence of maternal toxicity was observed as a decrease in weight gain for dams treated with 40 mg/kg/day dinocap. Maternal weight gain was reduced throughout dosing (Tables I through III). This effect was observed during all three gestational periods but was most severe during the last pregnancy (Table VI). For the first and third pregnancies, this decrease in weight gain persisted throughout pregnancy (Tables I and III). Evidence of maternal toxicity was not seen in the other treatment groups.

Neonatal Effects

Malformed neonates are frequently cannibalized by the dams. Since the dams generally delivered their litters at night and the litters were observed the following

morning, the observations made on postnatal day 1 probably underestimate the number of dead neonates and, thus, the total number of pups in a litter. This phenomenon may explain the difference in litter size between the control animals and those treated with dinocap at 40 mg/kg/d (Tables I and III). The litter loads of the remaining treatment groups were not different from those of control animals.

Considerable offspring mortality on postnatal days 1 and 3 was observed in the 40 mg/kg/day dinocap treatment group (Tables I through III). Increased neonatal mortality in the 20 mg/kg/day dinocap group was only seen on postnatal day 3 of the third litter (Table III). In many of the neonates that died postnatally, a "ballooned" abdomen was observed. This effect has been observed previously in dinocap-treated litters and was found to be associated with cleft palate. The "ballooning" results from a neonate swallowing air so that eventually the gastrointestinal tract is filled, distending the abdomen (Gray et.al., 1986).

Developmental toxicity in the two dinocap groups was also observed as a decrease in mean neonatal weight on postnatal day 1 (Tables I through III). This was observed in the third litter of the low dose group and all three litters of the high dose group. By postnatal day 3, however, in all but the second litter for the high dose group, there were no weight differences between dinocap-treated neonates and control neonates. This most likely is the result of the high mortality of severely affected pups, as noted above. All pups that survived through postnatal day 3 appeared normal.

Developmental toxicity increased by the third litter in the dinocap treated animals; that is, neonatal survival was significantly reduced in the last litter compared to

the first two litters (Tables V and VI). A previous study had hypothesized that the CD-1 mouse may metabolize dinocap differently than other species (Rogers, et. al., 1988). One possibility may be that exposure to dinocap induces synthesis of liver xenobiotic metabolizing enzymes, increasing the availability of a toxic metabolite. If these enzymes were induced over a period of time, this may account for a greater response with dosing during subsequent pregnancies. Frequently, the induction of liver enzymes may be observed as an increase in liver weight. Analysis of absolute and relative liver weights following completion of the third litter observations, however, did not show a difference between dinocap treated and control animals (Table IX).

Administration of all-trans retinoic acid at either 10 or 20 mg/kg/day significantly reduced neonatal survival on postnatal day 3 for all three litters in a dose dependent manner. A reduction in survival rate was also seen on postnatal day 1 in all three litters of the high dose group (Tables I through III). Similar to the dinocap treated animals, mean neonatal weights were slightly reduced on postnatal day 1 for the first two high dose litters, however, by postnatal day 3 all surviving pups appeared normal.

The "ballooning" gastrointestinal tracts observed in the dinocap animals were also observed in many of the neonates that died following the all-trans retinoic acid treatment. In addition, hind limb defects were observed in many of these animals. These observations correlate with the results of previous studies where cleft palate and limb defects were observed in other mouse strains following treatment with all-trans retinoic acid during similar gestational periods (Kochhar, et. al., 1984; Abbott, et.al., 1989; Abbott, et.al., 1990).

Interlitter Differences

The results of a rank test of independence are presented in Tables X through XIV and Figure I. Probability levels less than or equal to 0.05 indicate rank comparisons where the null hypothesis could be rejected; that is, the dams responded consistently from one litter to another.

In the control animals, a pattern of consistent reponse was seen for many variables in all three pairs of litter comparisons (Table X). Figure I illustrates this patterned response by variable for the three litter comparisons. For some variables, these results are not surprising. For example, few neonates died in the control litters, therefore, survival rates and the number of dead neonates per litter remained virtually constant.

A similar but less dramatic pattern was seen in the 10 mg/kg/day all-trans retinoic acid treated animals (Table XIII and Figure I). The remainder of the treatment groups exhibited more random patterns of response across litters with respect to specific variables as evidenced by probability levels less than 0.05 (Tables XI, XII, and XIV and Firgure I). This result was observed despite few differences in mean litter values (Tables IV through VIII).

Inter- Versus Intra-dam Variability

Intradam variability, as calculated for this study, includes variation specifically associated with the parental pair as well as variation in the litter. In addition to genetic variability, other factors that may account for the interdam variability include: experimental error and/or variation; differences in the amount of food in the stomach of

animals dosed orally; differences in the health status of the dams which may not be apparent to the investigator; and kinetic differences in litter load or position in the uterus. Experimental error and/or variation can be minimized by use of good laboratory practices, but can never be completely eliminated. All possible steps were taken to ensure a consistent environment for the animals including the random assignment of dams to a treatment group; the preparation of dosing solutions was assumed by one individual; and the dosing and observation of animals at a consistent time each day.

Since individual offspring measurements were not made in this study, the relative contribution of maternal versus litter variability to the "intradam" variability cannot be determined.

Likewise, since CD-1 male mice were used in breeding, the relative contribution of paternal variability could also not be determined. Each male was initially assigned five females, one for each treatment group, so that significant paternal variation would not be limited to one treatment group. To study the impacts of genetic variation associated with the sire on inter-litter difference the study would need to be repeated with males from an inbred strain since these animals are virtually genetically identical.

No intraclass correlation coefficient calculated was greater than 0.50 (Table XV). This indicates that intradam variability contributes more to the total variability observed than interdam variability. Interdam variability, though smaller than intradam variability, did appear to contribute substantially to overall variation. This was most easily seen in the control animals where the intraclass correlation coefficients ranged from 0.20 to 0.40. Similarily, intraclass correlation coefficients in this range were

observed for the endpoints evaluated on postnatal day 3 in the 10 mg/kg/d retinoic acid animals. These values generally decreased in the other treatment groups for most variables, though few were statistically significant from controls. The lack of statistical significance was primarily related to large confidence intervals associated with the intraclass correlation coefficients.

SUMMARY

The present study confirmed the results of previous experiments showing that dinocap and all-trans retinoic acid are teratogenic in CD-1 mice. Using a modification of an in vivo teratology screen that assesses postnatal growth and viability (Chernoff and Kavlock, 1982), developmental toxicity was observed as an increase in neonatal mortality by postnatal day 3 in all treatment groups in one (20 mg/kg/day dinocap; litter 3) or all three litters. In addition, an increase in neonatal mortality as well as a decrease in mean neonate weight were seen on postnatal day 1 in the majority of the two high dose litters. Malformations characteristic of treatment with these teratogens were also observed in some surviving neonates.

Individual maternal variation appeared to contribute significantly to the responses of untreated animals. A consistent response pattern was observed in untreated animals as evidence by consistent ranks across litters for specific endpoints. That is, a dam that had a large number of pups in one litter tended to have large second and third litters when compared to the other dams in the control group. Relatively large intraclass correlation coefficients observed for the control animals also indicate that interdam variation contributes significantly to interlitter differences.

The significance of individual maternal variability decreased when the dams were exposed to dinocap or all-trans retinoic acid. Specifically, the relative rank of a dam for a given variable within a treatment became more random and the intraclass correlation coefficients decreased, in some cases to less than 0.10. This finding was observed in two treatment groups (dinocap: 40 mg/kg/day; all-trans retinoic acid: 20

mg/kg/day) showing statistically significant differences in several endpoints when compared to controls using standard statistical test as well as a third treatment group (dinocap: 20 mg/kg/day) where most endpoints were not significantly different than controls. These data raise the possibility of an environmental exposure, in this case, a known developmental toxicant, masking the role of normal individual variation, including differences related to genetic variation.

REFERENCES

Abbott, B.D.; Harris, M.W. and Birnbaum, L.S. (1989). Etiology of retinoic acid-induced cleft palate varies with the embryonic stage. Teratology. 40:533-553.

Abbott, B.D.; Hill, L.G.; and Birnbaum, L.S. (1990). Processes involved in retinoic acid production of small embryonic palatal shelves and limb defects. Teratology. 41:299-310.

Biddle, F.G. (1981). The role of genetic studies in developmental toxicology. Developmental Toxicology (C.A. Kimmel and J. Buelke-Sam, eds). pp. 55-82.

Biddle, F.G. and Fraser, F.C. (1977). Maternal effects in experimental teratology. Handbook of Teratology: Volume III - Comparative, Maternal, and Epidemiologic Aspects (J.G. Wilson and F.C Fraser, eds.). pp. 3-33.

Birnbaum, L.S.; Harris, M.W.; Stocking, L.M.; Clark, A.M.; and Morrissey, R.E. (1989). Retinoic acid and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) selectively enhance teratogenesis in C57BL/6N mice. Toxicology and Applied Pharmacology. 98:487-500.

Brockes, S.P. (1989). Retinoids, homeobox genes, and limb morphogenesis. Nature, Lond. 332:850-853.

Chernoff, N. and Kavlock, R.J. (1982). An in vivo teratology screen utilizing pregnant mice. J Tox and Env Health. 10:541-550.

Donner, A. and Wells, G. (1986). A comparison of confidence interval methods for the intraclass correlation coefficient. Biometrics. 42:401-12.

Dourson, M.L. and Stara, J.F. (1983). Regulatory history and experimental support of uncertainty factors. Reg Tox and Pharmacology. 3:224-238.

Festing, M.F.W. (1990). Use of genetically heterogeneous rats and mice in toxicological researce: a personal perspective. Toxicology and Applied Pharmacology. 102:197-204.

Gill, T.J. (1980). The use of randomly bred and genetically defined animals in biomedical research. Americal Jornal of Pathology. 101(3) S21-32.

Geelen, J.A.G. (1979). Hypervitaminosis A induce teratogenesis. CRC Crit Rev Toxicol. 6:351-375.

Gray, L.E. and Kavlock, R.J. (1984). An extended evaluation of an in vivo teratology screen utilizing postnatal growth and viability in the mouse. Teratogenesis, Carcinogenesis, and Mutagenesis. 4:403-426.

Gray, L.E.; Rogers, J.M.; Kavlock, R.J.; Ostbly, J.S.; Ferrell, J.M.; and Gray, K.L. (1986). Prenatal exposure to the fungicide dinocap causes behavioral torticollis, ballooning, and cleft palate in mice, but not rats or hamsters. Teratogenesis Carcinog Mutagen. 6:33-43.

Hanson, D.K. and Hodes, M.E. (1983). Comparative teratogenicity of phenytoin among several inbred strains of mice. Teratology. 28:175-179.

Inouye, M. and Kajiwara, Y. (1990). Strain difference of the mouse in manifestation of hydrocephalus following prenatal methylmercury exposure. Teratology. 41:205-210.

Kalter, H. (1979). The history of the A family of mice and biology of its congential malformations. Teratology. 20:213-

Kavlock, R.J.; Short, R.D.; and Chernoff, N. (1987) Further evaluation of an in vivo teratology screen. Teratogenesis, Carcinogenesis, and Mutagenesis. 7:7-16.

Kochhar, D.M.; Penner, J.D.; and Tellone, C.I. (1984) Comparative teratogenic activities of two retinoids: effects on palate and limb development. Teratogenesis, Carcinogenesis, and Mutagenesis. 4:377-387.

Kurtz, C.P.; Baum, H.; and Swittenbank, C. (1970). Gas chromatographic determination of total active ingredient content of Karathane Technical and Karathane.

Lehmann, E.L. (1975). Nonparametrics: Statistical Methods Based on Ranks. Holden-Day, Inc., San Francisco. pp. 287-303.

Maden, M. (1985). Retinoids and the control of pattern in limb development and regenration. Trends in Genetics. 1:103-104.

Robertson, M. (1987). Towards a biochemistry of morphogenesis. Nature. 330:420-421.

Rogers, J.M.; Carver, B.; Gray, L.E.; Jr.; Gray, J.A.; and Kavlock, R.J. (1986). Teratogenic effects of the fungicide dinocap in the mouse. Teratogenesis Carcinog Mutagen. 6:375-81.

Rogers, J.M.; Gray. L.E., Jr.; Carver, B.; and Kavlock, R.J. (1987b). Developmental toxicity of dinocap in the mouse is not due to two isomers of the major active ingredients. Teratogenesis Carcinog Mutagen. 7:341-46.

SAS Institute, Inc. (1985). SAS Users Guide: Statistics. SAS Institute, Inc., Cary, NC.

Satre, M.A. and Kochhar, D.M. (1988). Endogenous concentrations of retinol and retinoic acid in embryonic mouse tissues and limb-buds. Teratology. 37:489.

Seller, M.J.; Perkins, K.J.; and Adenolfi, M. (1983). Differential response of heterozygous curly-tail mouse embryos to vitamin A teratogenesis depending on maternal genotype. Teratology. 28:123-129.

Swiger, L.A.; Harvey, W.R.; Everson, D.O.; and Gregory, K.E. (1964). The variance of intraclass correlation involving groups with one observation. Biometrics 20:818-826.

Thaller, C. and Eichele, G. (1988). Characterization of retinoid metabolism in the developing chick limb bud. Development. 103:47-483.

Thaller, C. and Eichele, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. Nature. 327: 625-628.

Tickle, C.; Alberts, B.; Wolpert, L. and Lee, J. (1982). Local application of retinoic acid to the limb bud mimics the action of the polarizing region. Nature. 296: 564-566.

U.S. Environmental Protection Agency (1987). Integrated Risk Information System - Supportive Documentation, Volume I. EPA 600/8-86-032a. Washigton D.C.

Table I - Litter 1
Maternal and Neonatal Effects of Dinocap and All-trans Retinoic Acid

Variable	Control	Dinocap 20 mg/kg/d	Dinocap 40 mg/kg/d	Retinoic Acid 10 mg/kg/d	Retinoic Acid 20 mg/kg/d
			POSTNATAL DAY 1		
Total Number Neonates/ Litter	11.35 ± 0.60[a]	11.36 ± 0.65	9.79 * ± 0.62	10.91 ± 0.55	10.21 0.49
Number Live Neonates/ Litter	11.19 ± 0.64	10.50 ± 0.70	8.33 ** ± 0.67	9.64 ± 0.68	7.64 ** ± 0.60
Number Dead Neonates/ Litter	0.15 ± 0.29	0.86 ± 0.32	1.46 ** ± 0.31	1.27 ± 0.42	2.57 ** ± 0.38
Survival Rate	0.95 ± 0.04	0.91 ± 0.04	0.85 ± 0.04	0.88 ± 0.05	0.74 ** ± 0.04
Mean Neonate Weight (grams)	1.61 ± 0.03	1.57 ± 0.03	1.43 ** ± 0.03	1.61 ± 0.03	1.52 * ± 0.03
			POSTNATAL DAY 3		
Number Live Neonates/ Litter	11.15 ± 0.59	9.68 ± 0.64	2.33 ** ± 0.61	6.09 ** ± 0.67	0.89 ** ± 0.59
Number Dead Neonates/ Litter	0.038 ± 0.47	0.82 ± 0.51	6.00 ** ± 0.49	3.54 ** ± 0.56	6.75 ** ± 0.50
Survival Rate	0.95 ± 0.05	0.84 ± 0.05	0.26 ** ± 0.05	0.55 ** ± 0.05	0.10 ** ± 0.05
Mean Neonate Weight (grams)	2.21 ± 0.05	2.21 ± 0.05	2.10 ± 0.06	2.42 ± 0.09	2.21 ± 0.12
			MATERNAL WEIGHT GAIN (grams)		
During Dosing (g.d. 10-13)	5.83 ± 0.40	5.94 ± 0.44	3.49 ** ± 0.42	------	------
During Dosing (g.d. 11-13)	3.94 ± 0.38	------	------	3.87 ± 0.39	3.41 ± 0.35
Total (g.d. 0-17)	25.57 ± 0.96	23.60 ± 1.04	21.62 ** ± 1.00	25.32 ± 1.04	25.69 ± 0.93

[a] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.
** Significantly different from controls, $p \leq 0.01$.

Table II - Litter 2
Maternal and Neonatal Effects of Dinocap and All-trans Retinoic Acid

Variable	Control	Dinocap 20 mg/kg/d	Dinocap 40 mg/kg/d	Retinoic Acid 10 mg/kg/d	Retinoic Acid 20 mg/kg/d
			POSTNATAL DAY1		
Total Number Neonates/ Litter	12.50 ± 0.63[a]	12.54 ± 0.69	11.67 ± 0.66	12.00 ± 0.70	12.07 ± 0.64
Number Live Neonates/ Litter	12.35 ± 0.76	11.91 ± 0.83	9.46 * ± 0.80	11.54 ± 0.82	9.18 ** ± 0.73
Number Dead Neonates/ Litter	0.15 ± 0.39	0.64 ± 0.43	2.21 ** ± 0.41	0.45 ± 0.35	2.56 ** ± 0.32
Survival Rate	0.97 ± 0.04	0.94 ± 0.04	0.80 ** ± 0.04	0.96 ± 0.04	0.78 ** ± 0.03
Mean Neonate Weight (grams)	1.63 ± 0.02	1.56 * ± 0.02	1.37 ** ± 0.02	1.65 ± 0.03	1.51 ** ± 0.03
			POSTNATAL DAY3		
Number Live Neonates/ Litter	12.35 ± 0.72	11.23 ± 0.79	1.67 ** ± 0.75	7.77 ** ± 0.76	1.00 ** ± 0.67
Number Dead Neonates/ Litter	0.12 ± 0.54	0.68 ± 0.59	7.79 ** ± 0.57	3.82 ** ± 0.64	8.18 ** ± 0.57
Survival Rate	0.97 ± 0.04	0.90 ± 0.05	0.15 ** ± 0.04	0.64 ** ± 0.04	0.09 ** ± 0.04
Mean Neonate Weight (grams)	2.18 ± 0.06	2.13 ± 0.06	1.90 * ± 0.09	2.35 * ± 0.06	1.97 * ± 0.09
			MATERNAL WEIGHT GAIN (grams)		
During Dosing (g.d. 10-13)	6.30 ± 0.41	6.39 ± 0.44	4.23 ** ± 0.42	------	------
During Dosing (g.d. 11-13)	4.25 ± 0.32	------	------	4.59 ± 0.35	4.39 ± 0.31
Total (g.d. 0-17)	24.68 ± 0.89	23.67 ± 0.97	22.41 ± 0.93	26.22 ± 1.08	26.81 ± 0.95

[a] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.
** Significantly diffferent from controls, $p \leq 0.01$.

Table III - Litter 3

Neonatal and Maternal Effects of Dinocap and All-trans Retinoic Acid

Variable	Control	Dinocap 20 mg/kg/d	Dinocap 40 mg/kg/d	Retinoic Acid 10 mg/kg/d	Retinoic Acid 20 mg/kg/d
			POSTNATAL DAY1		
Total Number Neonates/ Litter	12.73 ± 0.62[a]	12.91 ± 0.68	10.08 ** ± 0.65	11.45 ± 0.58	11.39 ± 0.51
Number Live Neonates/ Litter	12.69 ± 0.80	11.95 ± 0.87	6.71 ** ± 0.83	10.27 * ± 0.76	8.68 ** ± 0.67
Number Dead Neonates/ Litter	0.04 ± 0.48	0.95 ± 0.52	3.38 ** ± 0.49	1.18 ± 0.44	2.71 ** ± 0.39
Survival Rate	1.00 ± 0.05	0.93 ± 0.05	0.62 ** ± 0.05	0.88 ± 0.04	0.75 ** ± 0.04
Mean Neonate Weight (grams)	1.62 ± 0.04	1.52 ± 0.04	1.41 ** ± 0.04	1.61 ± 0.03	1.55 ± 0.03
			POSTNATAL DAY3		
Number Live Neonates/ Litter	12.62 ± 0.58	8.77 ** ± 0.63	1.04 ** ± 0.60	4.86 ** ± 0.67	1.43 ** ± 0.59
Number Dead Neonates/ Litter	0.077 ± 0.72	3.18 ** ± 0.78	5.67 ** ± 0.75	5.41 ** ± 0.65	7.25 ** ± 0.58
Survival Rate	0.99 ± 0.04	0.70 ** ± 0.04	0.12 ** ± 0.04	0.40 ** ± 0.04	0.12 ** ± 0.04
Mean Neonate Weight (grams)	2.18 ± 0.06	2.26 ± 0.06	2.24 ± 0.10	2.31 ± 0.08	2.17 ± 0.08
			MATERNAL WEIGHT GAIN (grams)		
During Dosing (g.d. 10-13)	6.46 ± 0.36	5.91 ± 0.39	1.90 ** ± 0.37	------	------
During Dosing (g.d. 11-13)	4.52 ± 0.23	------	------	3.97 ± 0.25	4.10 ± 0.22
Total (g.d. 0-17)	24.39 ± 0.87	24.41 ± 0.95	20.21 ** ± 0.91	24.62 ± 1.11	26.42 ± 1.00

[a] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.
** Significantly different from controls, $p \leq 0.01$.

Table IV

Neonatal and Maternal Effects in Control Animals

Variable	Litter 1	Litter 2	Litter 3
		POSTNATAL DAY 1	
Total Number Neonates/ Litter	11.35 ± 0.65[a]	12.50 ± 0.65	12.73 ± 0.65
Number Live Neonates/ Litter	11.19 ± 0.69	12.35 ± 0.69	12.69 ± 0.69
Number Dead Neonates/ Litter	0.15 ± 0.08	0.15 ± 0.08	0.04 ± 0.08
Survival Rate	0.95 ± 0.03	0.97 ± 0.03	1.00 ± 0.03
Mean Neonate Weight (grams)	1.61 ± 0.02	1.63 ± 0.02	1.62 0.02
		POSTNATAL DAY 3	
Number Live Neonates/ Litter	11.15 ± 0.70	12.35 ± 0.70	12.62 ± 0.70
Number Dead Neonates/ Litter	0.04 ± 0.07	0.12 ± 0.07	0.08 ± 0.07
Survival Rate	0.95 ± 0.03	0.97 ± 0.03	0.99 ± 0.03
Mean Neonate Weight (grams)	2.21 ± 0.05	2.18 ± 0.05	2.18 ± 0.05
		MATERNAL WEIGHT GAIN (grams)	
During Dosing (g.d. 10-13)	5.83 ± 0.38	6.30 ± 0.38	6.46 ± 0.38
During Dosing (g.d. 11-13)	3.94 ± 0.34	4.25 ± 0.32	4.52 ± 0.32
Total (g.d. 0-17)	25.57 ± 0.98	24.68 ± 0.98	24.39 ± 0.98

[a] Values are least squares means (± S.E.).

Table V

Neonatal and Maternal Effects of Dinocap (20 mg/kg/d)

Variable	Litter 1	Litter 2	Litter 3
		POSTNATAL DAY 1	
Total Number Neonates/ Litter	11.36 ± 0.60[a]	12.54 ± 0.60	12.91 ± 0.60
Number Live Neonates/ Litter	10.50 ± 0.71	11.91 ± 0.71	11.95 ± 0.71
Number Dead Neonates/ Litter	0.86 ± 0.44	0.64 ± 0.44	0.95 ± 0.44
Survival Rate	0.91 ± 0.04	0.94 ± 0.04	0.93 ± 0.04
Mean Neonate Weight (grams)	1.57 ± 0.03	1.56 ± 0.03	1.52 ± 0.03
		POSTNATAL DAY 3	
Number Live Neonates/ Litter	9.68 ± 0.72	11.23 ± 0.72 (3-*)	8.77 ± 0.72 (2-*)
Number Dead Neonates/ Litter	0.82 ± 0.51 (3-**)	0.68 ± 0.51 (3-**)	3.18 ± 0.51 (1-**; 2-**)
Survival Rate	0.84 ± 0.05 (3-*)	0.90 ± 0.05 (3-**)	0.70 ± 0.05 (2**)
Mean Neonate Weight (grams)	2.21 ± 0.06	2.13 ± 0.06	2.26 ± 0.06
		MATERNAL WEIGHT GAIN (grams)	
During Dosing (g.d. 10-13)	5.94 ± 0.39	6.39 ± 0.39	5.91 ± 0.39
Total (g.d. 0-17)	23.60 ± 0.92	23.67 ± 0.92	24.41 ± 0.92

[a] Values are least squares means (± S.E.).
* Significantly different from indicated litter, $p \leq 0.05$.
** Significantly different from indicated litter, $p \leq 0.01$.

Table VI

Neonatal and Maternal Effects of Dinocap (40 mg/kg/d)

Variable	Litter 1	Litter 2	Litter 3
		POSTNATAL DAY 1	
Total Number Neonates/ Litter	9.79 ± 0.66[a] (2-*)	11.67 ± 0.66 (1-*)	10.08 ± 0.66
Number Live Neonates/ Litter	8.33 ± 0.89	9.46 ± 0.89 (3-*)	6.71 ± 0.89 (2-*)
Number Dead Neonates/ Litter	1.46 ± 0.58 (3-*)	2.21 ± 0.58	3.38 ± 0.58 (1-*)
Survival Rate	0.85 ± 0.06 (3-**)	0.80 ± 0.06 (3-*)	0.62 ± 0.06 (1-**; 2-*)
Mean Neonate Weight (grams)	1.43 ± 0.04	1.37 ± 0.04	1.41 ± 0.04
		POSTNATAL DAY 3	
Number Live Neonates/ Litter	2.33 ± 0.55	1.67 ± 0.55	1.04 ± 0.55
Number Dead Neonates/ Litter	6.00 ± 0.95	7.79 ± 0.95	5.67 ± 0.95
Survival Rate	0.26 ± 0.06	0.15 ± 0.06	0.12 ± 0.06
Mean Neonate Weight (grams)	2.10 ± 0.10	1.90 ± 0.12	2.24 ± 0.12
		MATERNAL WEIGHT GAIN (grams)	
During Dosing (g.d. 10-13)	3.49 ± 0.44 (3-*)	4.23 ± 0.44 (3-**)	1.90 ± 0.44 (1-*; 2-**)
Total (g.d. 0-17)	21.62 ± 0.90	22.41 ± 0.90	20.21 ± 0.90

[a] Values are least squares means (± S.E.).
* Significantly different from indicated litter, $p \leq 0.05$.
** Significantly different from indicated litter, $p \leq 0.01$.

Table VII

Neonatal and Maternal Effects of All-trans Retinoic Acid (10 mg/kg/d)

Variable	Litter 1	Litter 2	Litter 3
		POSTNATAL DAY 1	
Total Number Neonates/ Litter	10.91 ± 0.58[a]	12.00 ± 0.58	11.45 ± 0.58
Number Live Neonates/ Litter	9.64 ± 0.68	11.54 ± 0.68	10.27 ± 0.68
Number Dead Neonates/ Litter	1.27 ± 0.35	0.45 ± 0.35	1.18 ± 0.35
Survival Rate	0.88 ± 0.03	0.96 ± 0.03	0.88 ± 0.03
Mean Neonate Weight (grams)	1.61 ± 0.04	1.65 ± 0.04	1.61 ± 0.04
		POSTNATAL DAY 3	
Number Live Neonates/ Litter	6.09 ± 0.91	7.77 ± 0.91 (3-*)	4.86 ± 0.91 (2-*)
Number Dead Neonates/ Litter	3.54 ± 0.75	3.82 ± 0.75	5.41 ± 0.75
Survival Rate	0.55 ± 0.07	0.64 ± 0.07 (3-*)	0.40 ± 0.07 (2-*)
Mean Neonate Weight (grams)	2.42 ± 0.08	2.35 ± 0.08	2.32 ± 0.09
		MATERNAL WEIGHT GAIN (grams)	
During Dosing (g.d. 11-13)	3.87 ± 0.39	4.59 ± 0.39	3.97 ± 0.39
Total (g.d. 0-17)	25.32 ± 1.23	26.22 ± 1.23	24.62 ± 1.23

[a] Values are least squares means (± S.E.).
* Significantly different from indicated litter, $p \leq 0.05$.

Table VIII

Neonatal and Materal Effects of All-trans Retinoic Acid (20 mg/kg/d)

Variable	Litter 1	Litter 2	Litter 3
		POSTNATAL DAY 1	
Total Number Neonates/ Litter	10.21 ± 0.49[a] (2-**)	12.07 ± 0.49 (1-**)	11.39 ± 0.49
Number Live Neonates/ Litter	7.64 ± 0.72	9.18 ± 0.72	8.68 ± 0.72
Number Dead Neonates/ Litter	2.57 ± 0.52	2.56 ± 0.53	2.71 ± 0.52
Survival Rate	0.74 ± 0.05	0.78 ± 0.05	0.75 ± 0.05
Mean Neonate Weight (grams)	1.52 ± 0.03	1.51 ± 0.03	1.55 ± 0.03
		POSTNATAL DAY 3	
Number Live Neonates/ Litter	0.89 ± 0.33	1.00 ± 0.33	1.43 ± 0.33
Number Dead Neonates/ Litter	6.75 ± 0.68	8.18 ± 0.68	7.25 ± 0.68
Survival Rate	0.10 ± 0.03	0.09 ± 0.03	0.12 ± 0.03
Mean Neonate Weight (grams)	2.21 ± 0.14	1.97 ± 0.14	2.17 ± 0.13
		MATERNAL WEIGHT GAIN (grams)	
During Dosing (g.d. 11-13)	3.41 ± 0.26 (2-**)	4.39 ± 0.23 (1-**; 3-*)	4.10 ± 0.23 (2-*)
Total (g.d. 0-17)	25.69 ± 0.84	26.81 ± 0.84	26.42 ± 0.85

[a] Values are least squares means (± S.E.).
* Significantly different from indicated litter, $p \leq 0.05$.
** Significantly different from indicated litter, $p \leq 0.01$.

Table IX

Liver-to-Body Weight Ratios[a]

Variable	Control	Dinocap (20 mg/kg/d)	Dinocap (40 mg/kg/d)
Liver/Body Weight	0.072 ± 0.0017[b]	0.071 ± 0.0020	0.073 ± 0.0018

[a] Liver and body weights were measured following observation of the third litter on postnatal day 3.

[b] Values are least squares means (± S.E.).

Table X

Rank Test of Independence - Control

	Litter 1 - Litter 2		Litter 1 - Litter 3		Litter 2 - Litter 3	
Variable	**N**	**Significance**	**N**	**Significance**	**N**	**Significance**
			POSTNATAL DAY 1			
Total Number Neonates/ Litter	26	0.0165	26	0.0029	26	0.0727
Number Live Neonates/ Litter	26	0.0191	26	0.0278	26	0.02418
Number Dead Neonates/ Litter	26	<0.0006	26	<0.0001	26	<0.0001
Survival Rate	26	0.0006	26	<0.0001	26	<0.0001
Mean Neonate Weight	25	0.1416	25	0.0124	25	0.4722
			POSTNATAL DAY 3			
Number Live Neonates/ Litter	26	0.02116	26	0.0024	26	0.0517
Number Dead Neonates/ Litter	26	<0.0001	26	<0.0001	26	<0.0001
Survival Rate	26	0.0131	26	0.0005	26	0.0016
Mean Neonate Weight	23	0.4346	23	0.0129	26	0.4921
			MATERNAL WEIGHT GAIN			
Dosing (g.d. 10-13)	26	0.0887	26	0.0003	26	0.0440
Dosing (g.d. 11-13)	24	0.7427	24	0.0003	26	0.3844
Total (g.d. 0-17)	26	0.0378	26	0.0153	26	0.0360

Table XI

Rank Test of Independence - Dinocap (20 mg/kg/d)

Variable	Litter 1 - Litter 2		Litter 1 - Litter 3		Litter 2 - Litter 3	
	N	Significance	N	Significance	N	Significance
			POSTNATAL DAY 1			
Total Number Neonates/ Litter	22	0.0531	22	0.3006	22	0.0005
Number Live Neonates/ Litter	22	0.4716	22	0.1521	22	0.0100
Number Dead Neonates/ Litter	22	0.0576	22	0.0248	22	0.0011
Survival Rate	22	0.0736	22	0.01088	22	0.0010
Mean Neonate Weight	21	0.7024	22	0.8776	21	0.0275
			POSTNATAL DAY 3			
Number Live Neonates/ Litter	22	0.3418	22	0.9269	22	0.2638
Number Dead Neonates/ Litter	22	0.1076	22	0.7395	22	0.0138
Survival Rate	22	0.7353	22	0.1555	22	0.0077
Mean Neonate Weight	20	0.5411	20	0.9037	20	0.0353
			MATERNAL WEIGHT GAIN			
Dosing (g.d. 10-13)	22	0.4670	22	0.5581	22	0.6135
Total (g.d. 0-17)	22	0.1498	22	0.1064	22	0.0030

Table XII

Rank Test of Independence - Dinocap (40 mg/kg/d)

Variable	Litter 1 - Litter 2		Litter 1 - Litter 3		Litter 2 - Litter 3	
	N	Significance	N	Significance	N	Significance
			POSTNATAL DAY 1			
Total Number Neonates/ Litter	24	0.4041	24	0.04957	24	0.2328
Number Live Neonates/ Litter	24	0.2883	24	0.02194	24	0.20685
Number Dead Neonates/ Litter	24	0.2765	24	0.12022	24	0.020165
Survival Rate	24	0.3429	24	0.20596	24	0.01694
Mean Neonate Weight	24	0.2051	21	0.3769	21	0.94071
			POSTNATAL DAY 3			
Number Live Neonates/ Litter	24	0.1743	24	0.00797	24	0.01246
Number Dead Neonates/ Litter	24	0.2376	24	0.006302	24	0.09537
Survival Rate	24	0.3054	24	0.013116	24	0.004886
Mean Neonate Weight	5	>0.9999	7	>0.9999	6	0.9632
			MATERNAL WEIGHT GAIN			
Dosing (g.d. 10-13)	24	0.01854	24	0.7572	24	0.4334
Total (g.d. 0-17)	24	0.3642	24	0.8262	24	0.5494

Table XIII

Rank Test of Independence - Retinoic Acid (10 mg/kg/d)

Variable	Litter 1 - Litter 2		Litter 1 - Litter 3		Litter 2 - Litter 3	
	N	Significance	N	Significance	N	Significance
			POSTNATAL DAY 1			
Total Number Neonates/ Litter	22	0.1056	22	0.7163	22	0.02930
Number Live Neonates/ Litter	22	0.006442	22	0.7159	22	0.1567
Number Dead Neonates/ Litter	22	0.06720	22	0.1593	22	0.0358
Survival Rate	22	0.04825	22	0.1640	22	0.0606
Mean Neonate Weight	22	0.001710	22	0.4024	22	0.1004
			POSTNATAL DAY 3			
Number Live Neonates/ Litter	22	0.01939	22	0.02724	22	0.001253
Number Dead Neonates/ Litter	22	0.01558	22	0.0705	22	0.008356
Survival Rate	22	0.00367	22	0.0133	22	0.009026
Mean Neonate Weight	18	0.1752	13	0.7095	14	0.8434
			MATERNAL WEIGHT GAIN			
Dosing (g.d. 11-13)	22	0.6380	22	0.7954	22	0.0849
Total (g.d. 0-17)	22	0.07888	22	0.09992	22	0.004362

Table XIV

Rank Test of Independence - Retinoic Acid (20 mg/kg/d)

Variable	Litter 1 - Litter 2		Litter 1 - Litter 3		Litter 2 - Litter 3	
	N	Significance	N	Significance	N	Significance
			POSTNATAL DAY 1			
Total Number Neonates/ Litter	27	0.3654	28	0.2132	27	0.0008
Number Live Neonates/ Litter	28	0.0296	28	0.1067	28	0.0936
Number Dead Neonates/ Litter	27	0.00109	28	0.0103	27	0.1208
Survival Rate	27	0.0005	28	0.0055	27	0.0772
Mean Neonate Weight	25	0.2829	24	0.6106	24	0.1403
			POSTNATAL DAY 3			
Number Live Neonates/ Litter	28	0.01082	28	0.0140	28	0.0530
Number Dead Neonates/ Litter	28	0.1451	28	0.1308	28	0.1956
Survival Rate	27	0.01745	28	0.0104	27	0.1348
Mean Neonate Weight	5	0.9074	5	>0.9999	5	0.9968
			MATERNAL WEIGHT GAIN			
Dosing (g.d. 11-13)	28	0.2461	28	0.0976	28	0.0094
Total (g.d. 0-17)	28	0.0033	27	0.0025	27	0.0154

Figure I

RANK TEST OF INDEPENDENCE

	Variable	Control 0 mg/kg/d	Dinocap 20 mg/kg/d	Dinocap 40 mg/kg/d	Retinoic Acid 10 mg/kg/d	Retinoic Acid 20 mg/kg/d
POSTNATAL DAY 1	Total #/Litter	■ ▲	●	▲	●	●
	# Live/Litter	■ ▲ ●	●	▲	■	■
	# Dead/Litter	■ ▲ ●	▲ ●	●	●	■ ▲
	Survival Rate	■ ▲ ●	▲ ●	●	■	■ ▲
	Mean Pup Wgt./Litter	▲	●		■	
POSTNATAL DAY 3	# Live/Litter	■ ▲		▲ ●	■ ▲ ●	■ ▲
	# Dead/Litter	■ ▲ ●	●	▲	■ ●	
	Survival Rate	■ ▲ ●	●	▲ ●	■ ▲ ●	■ ▲
	Mean Pup Wgt./Litter	▲	●			
MATERNAL WGT. GAIN	Dosing (g.d.10-13)	▲ ●		■		
	Dosing (g.d. 11-13)	▲				●
	Total (g.d. 0-17)	■ ▲ ●	●		●	■ ▲ ●

■ Litter 1 compared to Litter 2

▲ Litter 1 compared to Litter 3

● Litter 2 compared to Litter 3

Markers indicate where the null hypothesis was rejected; indicating that a consistent response was observed between the two litters.

Table XV

Intraclass Correlation Coefficients

Variable	Control	Dinocap 20 mg/kg/d	Dinocap 40 mg/kg/d	Retinoic Acid 10 mg/kg/d	Retinoic Acid 20 mg/kg/d
		POSTNATAL DAY 1			
Total Number Neonates/ Litter	0.3999	0.1823	0.1438	0.0760*	0.0896*
Number Live Neonates/ Litter	0.3816	0.1666	0.2310	0.1928	0.3103
Number Dead Neonates/ Litter	------	-----	0.1001	0.1645	0.2696
Survival Rate	------	------	0.0476	0.1218	0.2950
Mean Neonate Weight	0.2031	-----	------	0.3223	0.0484
		POSTNATAL DAY3			
Number Live Neonates/ Litter	0.3697	0.0247*	0.0866	0.4070	0.2033
Number Dead Neonates/ Litter	0.3710	------	0.2741	0.3190	0.1412
Survival Rate	------	0.0288	0.1242	0.4072	0.2061
Mean Neonate Weight (grams)	0.1994	0.1386	------	0.2974	------
		MATERNAL WEIGHT GAIN			
During Dosing (g.d. 10-13)	0.4047	0.0440*	0.0040*	------	------
During Dosing (g.d. 11-13)	0.2450	------	------	0.0063	0.1224
Total (g.d. 0-17)	0.3799	0.1396	0.3050	0.1463	0.4626

* Significantly different from controls, $p \leq 0.05$.

APPENDIX - PILOT STUDIES

BACKGROUND

Five chemicals were chosen for the initial study. These chemicals were dinocap, nitrofen, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), valproic acid, and all-trans retinoic acid. All of the chemicals are known developmental toxicants in the mouse. The first pilot study assessed all five chemicals using four or five treatment groups. The second pilot study reassessed three of the chemicals using three treatment groups. Dose levels were selected based upon results of previous studies conducted in the Environmental Protection Agency's Health Effects Research Laboratory or reported values in the literature. The dose levels were selected to produce defects that were readily observable and compatible with neonatal life for at least the first day after birth.

METHODS

Timed pregnant CD-1 mice (Charles River) were received on day 3 of gestation. Day 1 of gestation was recorded upon demonstration of a sperm plug. Each animal was housed individually and kept in a temperature-controlled (20-24° C) bioclean room with a 12-h light/dark cycle. They were fed commercial lab diet and water ad libitum. The mice were assigned to dose groups such that the mean weight and variance were similar in all dose groups, with equal animals per group. All chemicals were administered in 0.2 ml of corn oil by gastric intubation for 3 to 4 days during gestation days 8 through 13 (see Tables A-I through A-VIII for dosing days and dosage levels for specific chemicals). Dosing solutions were corrected for chemical purity and prepared based upon average maternal weight on day 8.

Endpoints were evaluated following an in vivo teratology screening test (Chernoff and Kavlock, 1982). These endpoints included: maternal body weight gain; evidence of maternal toxicity; evidence of developmental effects, and offspring viability and growth through postnatal day 3.

RESULTS AND DISCUSSION

The results of the first and second pilot studies are summarized in Tables A-I through A-V and A-VI through A-VIII, respectively. Evidence of maternal toxicity was seen as a decrease in body weight gain for animals treated with three of the chemicals. This was observed in seven treatment groups as follows: 2,4,5-T at 100, 200, and 400 mg/kg/day for gestation days 10 through 13 (Table A-V); nitrofen at 200 and 250 mg/kg/day for gestation days 8 through 11 (Table A-VI); and dinocap at 60 and 80 mg/kg/day for gestation days 10 through 13 (Table A-VII). Maternal toxicity was also observed as the death of two dams treated with 80 mg/kg/day dinocap on gestation days 10 through 13 (Table A-VII).

Maternal treatment with dinocap at 40 mg/kg/day during gestation days 11 through 13 caused an increase in neonatal death and an increase in mean pup weight by postnatal day 3. No effects were seen at dose levels of 10, 20, and 30 mg/kg/day (Table I). In the second pilot study, the dosing period was extended to include day 10 of gestation and the maternal dose was increased. These changes resulted in an increase in neonatal death per litter on postnatal day 1 (at 40 and 80 mg/kg/day), a decrease in mean neonate weight on postnatal days 1 and 3 (40, 60, and 80 mg/kg/day) and an

increase in neonatal death per litter on postnatal day 3 (40, 60, and 80 mg/kg/day) (Table VII).

In the first pilot, all-trans retinoic acid given to pregnant CD-1 mice on days 11 through 13 of gestation resulted in a decrease in the number of live neonates observed per litter on postnatal day 3 (25 and 50 mg/kg/day) and, thus, an increase in the number of dead neonates per litter also on postnatal day 3 (12.5, 25, and 50 mg/kg/day) (Table A-II). Similarily, results from the second pilot study showed a decrease in neonatal survival for all three treatment groups (12.5, 18.75, and 25 mg/kg/day) (Table A-VIII).

Maternal treatment with valproic acid at 300, 400, 500, and 600 mg/kg/day during days 11 through 13 of gestation produced no statistically significant differences in treated compared to control animals (Table A-III).

Nitrofen (250 mg/kg/day) given to dams on days 8 through 11 of gestation in the first pilot study produced a decrease in the number of live neonates per litter and, thus, an increase in the number of dead neonates per litter observed on postnatal day 3 (Table A-IV). In the second pilot study, differences between treated versus control animals included an increase in the number of dead neonates per litter on postnatal days 1 and 3 (200 mg/kg/day), a decrease in mean pup weight on postnatal days 1 (200 and 225 mg/kg/day), an increase in neonatal death per litter on postnatal day 3 (200, 225, and 250 mg/kg/day) and a decrease in mean neonate weight on postnatal day 3 (200, 225, and 250 mg/kg/day) (Table A-VI).

The effects of maternal dosing with 2,4,5-T during gestation days 10 through 13 included an increase in mean neonatal weight on postnatal days 1 and 3 in the 200 mg/kg/day treatment group but, a decrease in this parameter on postnatal days 1 and 3 for animals dosed at 400 mg/kg/day. A decrease of neonatal survival as evidenced by a decrease in the number of live neonates per litter and an increase in the number of dead neonates on postnatal day 3 was also observed at the highest dose (400 mg/kg/day) (Table A-V).

Based upon the results of the two pilot studies, two chemicals were chosen for further study using the multiple breeding protocol presented in the main text of this report. These chemicals and associated dosing regimes were dinocap at 20 and 40 mg/kg/day on days 10 through 13 of gestation and all-trans retinoic acid at 10 and 20 mg/kg/day on days 11 through 13 of gestation. The treatment levels were selected to produce minimal or no maternal toxicity. The two dosage levels per chemical were chosen such that approximately 25 to 35 percent of the litters would be affected at the low dose and approximately 50 to 75 percent of the litters would be affected at the high dose.

Table A-I

Pilot Study I

Maternal and Neonatal Effects of Dinocap

Maternal Dose (mg/kg/day)[a]

Parameter	0	10	20	30	40
			MATERNAL EFFECTS		
Number Pregnant	12	2	3	3	3
Number Not Pregnant	2	3	2	2	2
Maternal Weight Gain (grams) [g.d. 11-17]	19.29 ± 1.63[b]	18.85 ± 3.99	15.13 + 3.26	12.50 ± 3.26	14.10 ± 3.26
			LITTER EFFECTS - POSTNATAL DAY 1		
Live Neonates/ Litter	10.92 ± 1.14	10.50 ± 2.78	9.33 ± 2.27	8.00 ± 2.27	8.33 +2.27
Dead Neonates/ Litter	0	0	0	0	0
Mean Neonate weight (g)	1.63 ± 0.05	1.62 ± 0.12	1.67 ± 0.10	1.76 ± 0.10	1.85 ± 0.10
			LITTER EFFECTS - POSTNATAL DAY 3		
Live Neonates/ Litter	10.92 ± 1.14	11.00 ± 2.81	9.33 ± 2.29	8.00 ± 2.29	8.00 ± 2.29
Dead Neonates/ Litter	0 ± 0.06	0 ± 0.14	0 ± 0.11	0 ± 0.11	0.33 ± 0.11 *
Mean Neonate Weight (g)	2.32 ± 0.15	2.32 ± 0.37	2.50 ± 0.30	2.85 ± 0.30	3.05 ± 0.30 *

[a] Dosed days 11 throught 13 of gestation.
[b] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.

Table A-II

Pilot Study I

Maternal and Neonatal Effects of All-Trans Retinoic Acid

Parameter	Maternal Dose (mg/kg/day)[a] 0	6.25	12.5	25	50
			MATERNAL EFFECTS		
Number Pregnant	12	1	3	3	1
Number Not Pregnant	2	4	2	2	4
Maternal Weight Gain (grams) [g.d. 11-17]	19.29 ± 1.68[b]	15.90 ± 5.84	17.57 + 3.37	15.13 ± 3.37	19.80 ± 5.84
			LITTER EFFECTS - POSTNATAL DAY 1		
Live Neonates/ Litter	10.92 ± 1.00	10.00 ± 3.46	11.33 ± 2.00	8.00 ± 2.00	9.00 ± 3.46
Dead Neonates/ Litter	0	0	0	0	0
Mean Neonate Weight (g)	1.63 ± 0.05	1.62 ± 0.16	1.60 ± 0.09	1.67 ± 0.09	1.46 ± 0.16
			LITTER EFFECTS - POSTNATAL DAY 3		
Live Neonates/ Litter	10.92 ± 0.95	10.00 ± 3.29	10.33 ± 1.90	4.33 ± 1.90	0 ± 3.29*
Dead Neonates/ Litter	0 ± 0.27	0 ± 0.94	1.33 ± 0.54 *	3.67 ± 0.54 *	9.00 ± 0.94 *
Mean Neonate Weight (g)	2.32 ± 0.11	2.44 ± 0.38	2.45 ± 0.22	2.57 ± 0.22	-----

[a] Dosed days 11 throught 13 of gestation.
[b] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.

Table A-III

Pilot Study I

Maternal and Neonatal Effects of Valproic Acid

Maternal Dose (mg/kg/day)[a]

Parameter	0	300	400	500	600
			MATERNAL EFFECTS		
Number Pregnant	12	3	3	3	3
Number Not Pregnant	2	2	2	2	2
Maternal Weight Gain (grams) [g.d. 11-17]	19.29 ± 1.58[b]	16.47 ± 3.15	17.40 + 3.15	15.77 ± 3.15	12.57 ± 3.15
			LITTER EFFECTS - POSTNATAL DAY 1		
Live Neonates/ Litter	10.92 ± 1.06	11.00 ± 2.12	12.00 ± 2.12	11.33 ± 2.12	8.67 ± 2.12
Dead Neonates/ Litter	0	0	0	0	0
Mean Neonate Weight (g)	1.63 ± 0.04	1.49 ± 0.09	1.43 ± 0.09	1.46 ± 0.09	1.62 ± 2.15
			LITTER EFFECTS - POSTNATAL DAY 3		
Live Neonates/ Litter	10.92 ± 1.07	10.33 ± 2.15	12.00 ± 2.15	11.33 ± 2.15	8.67 ± 2.15
Dead Neonates/ Litter	0 ± 0.11	0.67 ± 0.22 *	0 ± 0.22	0 ± 0.22	0 ± 0.22
Mean Neonate Weight (g)	2.32 ± 0.10	2.03 ± 0.20	1.89 ± 0.20	2.00 ± 0.20	2.34 ± 0.20 *

[a] Dosed days 11 throught 13 of gestation.
[b] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.

Table A-IV

Pilot Study I

Maternal and Neonatal Effects of Nitrofen

Maternal Dose (mg/kg/day)[a]

Parameter	0	50	100	150	200	250
			MATERNAL EFFECTS			
Number Pregnant	12	4	4	3	3	2
Number Not Pregnant	2	1	1	2	2	3
Maternal Weight Gain (grams) [g.d. 8-17]	19.29 ± 1.40[b]	22.48 ± 2.43	21.30 ± 2.43	18.17 ± 2.81	23.17 ± 2.81	20.65 ± 3.44
			LITTER EFFECTS - POSTNATAL DAY 1			
Live Neonates/ Litter	10.92 ± 0.94	11.75 ± 1.63	10.75 ± 1.63	8.67 ± 1.88	10.33 ± 1.88	9.00 ± 2.30
Dead Neonates/ Litter	0	0	0	0	0	0
Mean Neonate Weight (g)	1.63 ± 0.04	1.50 ± 0.07	1.49 ± 0.07	1.52 ± 0.08	1.52 ± 0.08	1.59 ± 0.10
			LITTER EFFECTS - POSTNATAL DAY 3			
Live Neonates/ Litter	10.92 ± 0.88	11.50 ± 1.52	10.75 ± 1.52	8.33 ± 1.76	9.00 ± 1.76	4.50 ± 2.15 *
Dead Neonates/ Litter	0 ± 0.43	0.25 ± 0.74	0 ± 0.74	0.67 ± 0.86	1.33 ± 0.86	4.50 ± 1.05 *
Mean Neonate Weight (g)	2.32 ± 0.09	2.10 ± 0.16	2.20 ± 0.16	2.10 ± 0.19	2.23 ± 0.19	2.24 ± 0.23

[a] Dosed days 8 through 11 of gestation.
[b] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.

Table A-V

Pilot Study I

Maternal and Neonatal Effects of 2,4,5-T

Maternal Dose (mg/kg/day)[a]

Parameter	0	50	100	200	300	400
			MATERNAL EFFECTS			
Number Pregnant	12	4	3	4	3	3
Number Not Pregnant	2	1	2	1	2	2
Maternal Weight Gain (g) [g.d. 10-17]	19.29 ±2.97[b]	19.07 ± 2.97	12.33 ±1.48*	13.20 ±2.97*	14.57 ± 2.97	10.43 ±2.57*
			LITTER EFFECTS - POSTNATAL DAY 1			
Live Neonates/ Litter	10.92 ± 1.13	8.50 ± 1.96	7.67 ± 2.27	7.25 ± 1.96	6.00 ± 2.27	6.00 ± 2.27
Dead Neonates/ Litter	0 ± 0.65	2.50 ± 1.13	0 ± 1.31	0 ± 1.31	2.67 ± 1.31	0.33 ± 1.31
Mean Neonate Weight (g)	1.63 ± 0.04	1.56 ± 0.08	1.79 ± 0.08	1.82 ± 0.07*	1.64 ± 0.08	1.27 ± 0.08*
			LITTER EFFECTS - POSTNATAL DAY 3			
Live Neonates/ Litter	10.92 ± 1.11	8.50 ± 1.92	7.67 ± 2.21	7.25 ± 1.92	6.00 ± 2.21	2.23 ± 2.21 *
Dead Neonates/ Litter	0 ± 0.22	0 ± 0.44	0 ± 0.44	0 ± 0.38	0 ± 0.44	3.67 ± 0.44 *
Mean Neonate Weight (g)	2.32 ± 0.09	2.08 ± 0.19	2.70 ± 0.19	2.93 ± 0.16*	2.55 ± 0.19	2.44 ± 0.33

[a] Dosed days 10 through 13 of gestation.
[b] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.

Table A-VI

Pilot Study II

Maternal and Neonatal Effects of Nitrofen

Parameter	Maternal Dose (mg/kg/day)[a] 0	200	225	250
		MATERNAL EFFECTS		
Number Pregnant	11	6	5	5
Number Not Pregnant	7	3	4	4
Maternal Weight Gain (g) [g.d. 8 -11]	10.30 ± 0.90[b]	6.77 ± 1.22 *	7.32 ± 1.34	4.26 ± 1.34 *
		LITTER EFFECTS - POSTNATAL DAY 1		
Live Neonates/Litter	8.27 ± 1.19	7.50 ± 1.62	10.40 ± 1.77	5.60 ± 1.77
Dead Neonates/Litter	0.27 ± 0.40	1.67 ± 0.54 *	1.00 ± 0.60	0.60 ± 0.60
Mean Neonate Weight (g)	1.69 ± 0.06	1.38 ± 0.07 *	1.42 ± 0.08 *	1.46 ± 0.10
		LITTER EFFECTS - POSTNATAL DAY 3		
Live Neonates/Litter	9.40 ± 1.08	3.83 ± 1.40 *	6.40 ± 1.53	6.00 ± 2.00
Dead Neonates/Litter	0 ± 0.46	3.67 ± 0.59 *	4.00 ± 0.65 *	3.83 ± 0.84 *
Mean Neonate Weight (g)	2.27 ± 0.08	1.94 ± 0.11 *	1.78 ± 0.11 *	1.71 ± 0.14 *

[a] Dosed days 8 through 11 of gestation.
[b] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.

Table A-VII

Pilot Study II

Maternal and Neonatal Effects of Dinocap

Parameter	Maternal Dose (mg/kg/day)[a] 0	40	60	80
MATERNAL EFFECTS				
Number Pregnant	11	5	6	3
Number Not Pregnant	7	4	3	4
Number Dead	0	0	0	2
Maternal Weight Gain (g) [g.d. 10-13]	10.30 ± 0.93[b]	8.54 ± 1.38	5.90 ± 1.25 *	7.00 ± 1.77 *
LITTER EFFECTS - POSTNATAL DAY 1				
Live Neonates/Litter	8.27 ± 1.31	6.40 ± 1.95	6.33 ± 1.78	5.33 ± 2.52
Dead Neonates/Litter	0.27 ± 0.60	4.00 ± 0.89 *	1.83 ± 0.81	3.00 ± 1.15 *
Mean Neonate Weight (g)	1.69 ± 0.04	1.40 ± 0.06 *	1.29 ± 0.07 *	1.20 ± 0.08 *
LITTER EFFECTS - POSTNATAL DAY 3				
Live Neonates/Litter	9.40 ± 0.99	1.80 ± 1.41 *	0.50 ± 1.57	0 ± 1.82 *
Dead Neonates/Litter	0 ± 0.64	4.60 ± 0.90 *	9.00 ± 1.01 *	5.33 ± 1.17 *
Mean Neonate Weight (g)	2.27 ± 0.10	2.09 ± 0.23	1.65 ± 0.32	----

[a] Dosed days 10 through 13 of gestation.
[b] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.

Table A-VIII

Pilot Study II

Maternal and Neonatal Effects of All-trans Retinoic Acid

Parameter	Maternal Dose (mg/kg/day)[a] 0	12.5	18.75	25
MATERNAL EFFECTS				
Number Pregnant	11	7	8	5
Number Not Pregnant	7	3	1	3
Maternal Weight Gain (g) [g.d. 11-13]	10.30 ± 0.87[b]	11.21 ± 1.09	10.22 ± 1.02	11.28 ± 1.28
LITTER EFFECTS - POSTNATAL DAY 1				
Live Neonates/Litter	8.27 ± 1.15	8.00 ± 1.45	8.38 ± 1.35	9.40 ± 1.71
Dead Neonates/Litter	0.27 ± 0.46	1.43 ± 0.58	0.88 ± 0.54	0.80 ± 0.69
Mean Neonate Weight (g)	1.69 ± 0.05	1.67 ± 0.06	1.61 ± 0.06	1.52 ± 0.07
LITTER EFFECTS - POSTNATAL DAY 3				
Live Neonates/Litter	9.40 ± 1.08	4.57 ± 1.29 *	3.62 ± 1.21 *	0.20 ± 1.53 *
Dead Neonates/Litter	0 ± 0.78	3.43 ± 0.93 *	4.75 ± 0.87 *	9.20 ± 1.10 *
Mean Neonate weight (g)	2.27 ± 0.11	2.38 ± 0.14	2.16 ± 0.15	1.60 ± 0.36

[a] Dosed days 11 through 13 of gestation.
[b] Values are least squares means (± S.E.).
* Significantly different from controls, $p \leq 0.05$.