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Paternal Effects from Methamidophos Administration in Mice

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In this study, the mouse was used to evaluate paternal germline exposure to the organophosphate methamidophos for its potential to produce adverse effects on spermatozoa and in the offspring. There have been reports that organophosphate exposure can increase abnormal sperm morphology in mice. However, effects transmitted to the offspring following paternal exposure have not been reported previously. The maximum tolerated dose (MTD) was 7.5 mg kg^{-1} body weight and this dose resulted in no deaths, although blood plasma cholinesterase activity was still decreased. Males were euthanized 4 weeks after an acute intraperitoneal injection of methamidophos (0.5, 3.75, 5.0, and 7.5 mg kg^{-1} body wt) and the number of spermatids per gram testes and sperm morphology were analyzed. In this study, abnormal sperm morphology on a per group basis exhibited a dose–response significantly related to increased methamidophos exposure as indicated by regression analysis and a nested ANOVA ($p < 0.0001$). Preimplantation embryos that were conceived 6 weeks after paternal methamidophos exposure (5 mg kg^{-1} body wt) exhibited a significant increase in cleavage arrest. Fertility of males was also affected as shown by a decrease in the number of two- to four-cell embryos per male (postexposure week 6) and an increase in the number of degenerated embryos (postexposure weeks 4–6). We conclude that methamidophos may have the potential to produce transmissible adverse embryonic effects following an acute paternal germline exposure. © 2000 Academic Press

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Organophosphates have been reported to induce an increase in abnormal sperm morphology (Jayashree *et al.*, 1994; Wyrobek and Bruce, 1975; Mathew *et al.* 1992; Behera *et al.*, 1989) and to affect other sperm characteristics, including semen quality, in humans (Padungtod *et al.*, 1999). While abnormal sperm morphology has been used as an indirect endpoint for genetic changes in sperm following paternal exposures (Wyrobek *et al.*, 1983), these compounds have also been shown to increase other endpoints that are direct indicators of genetic damage such as chromosomal aberrations and micronuclei in bone marrow cells in mice (Jayashree *et al.*, 1994) and aneuploidy in human spermatozoa (Padungtod *et al.*, 1999). These observations are consistent with the hypothesis that paternal methamidophos exposure may result in transmitted effects that have the potential of decreasing the viability of the offspring.

The organophosphate methamidophos (*O,S,*-dimethyl phosphoramidothiolate, MW 141.12), marketed as Monitor or Tamaron, has a wide range of insecticidal actions (Fig. 1). It is used on crops such as brassica, cotton, tobacco, sugar beet, lettuce, potatoes, and tree fruits (WHO, 1993). In addition to its anticholinergic effects, this pesticide is more commonly known for its induction of organophosphate-induced delayed (poly)-neuropathy (OPIDN) in experimental animals (WHO, 1993; Khasawinah *et al.*, 1979). The anticholinesterase activity of methamidophos induces a decrease in plasma cholinesterase activity in mice (Zayed *et al.*, 1984). This inhibition of plasma cholinesterase by methamidophos can be used as a suitable index to monitor exposure to organophosphates (Zayed *et al.*, 1984). However, little is known about the effects of this organophosphate, if any, on mammalian reproduction or its potential for producing transmitted effects in offspring from paternal exposures.

Exposure to methamidophos has been correlated with an increase in the percentage of micronuclei in mouse hematopoietic cells *in vivo* and *in vitro* (Amer and Sayed, 1987), induction of sister chromatid exchange in mouse bone marrow cells *in vivo* and cultured mouse spleen cells *in vitro* (Amer and Sayed, 1987), and an induction of the alkylation of guanine in mice *in vivo* (Zayed and Mahdi, 1987). Collectively, these observations are consistent with the hypothesis that methamidophos has the capacity to induce genetic changes in spermatogenic cells and transmitted effects in the offspring following paternal exposure. In the following study, we tested this hypothesis using an increase in abnormal sperm morphology as an indirect measure of potential genetic changes in spermatozoa and impaired cell proliferation during preimplantation development *in vitro* as a measure of transmitted effects to the offspring.

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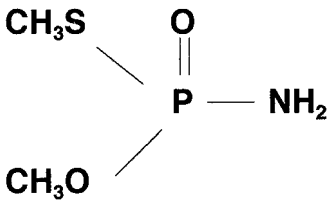

FIG. 1. Chemical structure of methamidophos (*O,S*-dimethyl phosphoamidothiolate) (Merck and Co., 1983).

METHODS

Animals

CD1 outbred male mice (Charles River), 8–10 weeks age, were used throughout this study. Superovulated (see below) CD1 outbred females, 7–8 weeks old, were used for breeding.

Males were stratified by weight and assigned randomly to treatment and control groups.

Range-Finding Assays

The range-finding assays consisted of two preliminary runs and one definitive range-finding assay to determine a lethal dose and a maximum tolerated dose (MTD). The MTD was defined as the dose that would not result in any deaths and no greater than 10% loss of body weight for a period of 4 weeks following administration (Generoso and Piergorsch, 1993). To monitor the exposure of the animals to the methamidophos, blood samples were taken for plasma cholinesterase measurements.

Three range-finding studies were done using ip injections of methamidophos to determine the MTD of methamidophos.

Study 1. Three dose groups consisting of four males each were given a single ip injection of methamidophos. The doses used were 15.0, 2.5, and 0.5 mg kg^{-1} body wt. One group of four males was given a sham injection as a control.

Study 2. Three groups of two males each were given a single ip injection of 10.0, 5.0, and 1.0 mg kg^{-1} body wt of methamidophos

Study 3. The doses used for this study were based on preliminary observations from Studies 1 and 2 in which doses lower than 2.5 mg kg^{-1} body wt did not produce significant changes in sperm morphology and doses of 10 and 15 mg kg^{-1} body wt resulted in death. A new dose of 7.5 mg kg^{-1} body wt was introduced with this study. This dose was chosen because the lethal dose by ip injection appeared to be between 15 and 10 mg kg^{-1} body wt. Assuming that the lethal dose was 15 mg kg^{-1} body wt, 7.5 mg kg^{-1} body wt is half of the putative lethal dose. Since the 10 mg kg^{-1} body wt dose was lethal and the 5.0 mg kg^{-1} body wt dose was nonlethal, a dose of 7.5 mg kg^{-1} body wt was tested as a potential MTD dose. Therefore, we studied a range of doses (mg kg^{-1} body wt) of 7.5 (putative MTD), 5.0 ($\frac{2}{3}$ MTD), 3.75 ($\frac{1}{2}$ MTD), and 0.5 ($\frac{1}{15}$ MTD) to determine whether a dose–response for abnormal sperm morphology might be observed, as previously reported for induction of cytogenetic effects in somatic cells (Amer and Sayed, 1987). Five to six males were used per group and these same males were also tested for number of spermatids per gram testes and abnormal sperm morphology.

Blood Sampling

Baseline blood samples were taken from the tails of control and treated mice at 1 to 1.5 weeks prior to treatment (data not shown). Postdose samples were drawn 24 h after treatment. Blood was collected in heparinized microfuge tubes and plasma was separated from blood cells by centrifugation at 1000*g*. Supernatant plasma was collected and stored frozen at 70°C until assayed for cholinesterase activity.

Cholinesterase Activity Measurement

Plasma acetylcholinesterase activity levels were measured as a biomarker to verify that methamidophos had induced a physiological response by inhibiting cholinesterase activity (Zayed *et al.*, 1984).

Plasma acetylcholinesterase activity was measured by the method of Ellman *et al.* (1961), modified for a 96-well microplate assay (Wilson *et al.*, 1996). The substrate was 1.0 mM acetylthiocholine iodide. Plasma samples were diluted $\frac{1}{50}$ with the assay buffer (0.1 M sodium phosphate, pH 8.0). The diluted plasma was assayed in triplicate using 30-μl aliquots in a final volume of 320 μl. Absorbance at 410 nm was read six times at 2-min intervals with a microplate reader (Biotek Instruments Model EL 340). Cholinesterase activity was calculated as follows: ChE activity (μmol/min per ml) = [sample A410 − blank A410] (OD/min) × 26.4 (nmol/O.D)/sample volume (ml)/dilution/1000 (nmol/μmol), where OD is the optical density and 26.4 nmol/OD is a constant derived from a standard curve of interaction of free sulfhydryl with the color reagent dithobisnitrobenzoate.

Cholinesterase activity was measured on males in all three range-finding studies. Blood from two animals was not analyzed due to insufficient sample. All males were weighed to monitor for weight loss.

In the studies on preimplantation embryos, a random sampling of three to five animals was evaluated from the control and dose groups for cholinesterase activity to confirm that physiological exposure to methamidophos had occurred.

Spermatid Quantitation and Sperm Morphology

Groups of five or six age-matched CD1 male mice (8 weeks old) were treated with an acute ip injection of one of the following doses; 0.5, 3.75, 5.0, or 7.5 mg kg^{-1} body wt doses of methamidophos (Chem Service, West Chester, PA) in 0.1 ml saline. A control group of five age-matched males was treated with a vehicle-injection of 0.1 saline. Males were euthanized at 4 weeks after methamidophos dose and were evaluated for mean number of spermatids per gram testes and sperm morphology (Wyrobek and Bruce, 1975).

Both testes were weighed and homogenized separately in 2.0 ml saline–Triton (0.9% NaCl and 0.05% Triton X-100). Spermatids per gram testes was determined by counting the number of spermatids in testicular homogenate and dividing by testis weight (g).

Cauda epididymal spermatozoa were collected by placing the epididymis into 2.0 ml of TYH culture medium (Toyoda *et al.*, 1971) containing 4% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and minced with scissors. Spermatozoa were allowed to swim out of the minced tissue for 20 min at 37°C in a humidified incubator filled with 5% CO_2 in air. Slides were made of caudal spermatozoa and were air dried, fixed with ethanol, and then stained with hematoxylin (Fisher Diagnostics, Lawn, NJ). Morphology was assessed using a light microscope (1000×). Ten slides were made per animal and 200 spermatozoa were counted per slide for a total of 2000 sperm per animal. The scorers were "blinded" as to the identity of the samples being evaluated. Spermatozoa were categorized into two groups according to normal or abnormal morphology. The abnormal group was further categorized into 11 subcategories: amorphous, acrosome, truncated, calyculate, collapsed, triangular, giant, hammer, tail, inverted, and double headed (Fig. 2) (Kot and Handel, 1987; Pogany and Balhorn, 1992; Burruel *et al.*, 1996). Before assessing sperm, wet mount preparations were compared to stained slides to ensure that sperm head categories were not altered by the dehydration process. No discrepancies were observed.

Postfertilization Endpoints

These endpoints were obtained from preimplantation embryos and consisted of the number of normal two- to four-cell embryos recovered and the proportion of degenerating embryos recovered per male. Also, an MTD dose from chemical mutagens has the potential to decrease preimplantation embryo number by at least 20% (Generoso *et al.*, 1975, 1985); therefore, to ensure that an adequate number of embryos were collected, the 5.0 mg kg^{-1} body wt was

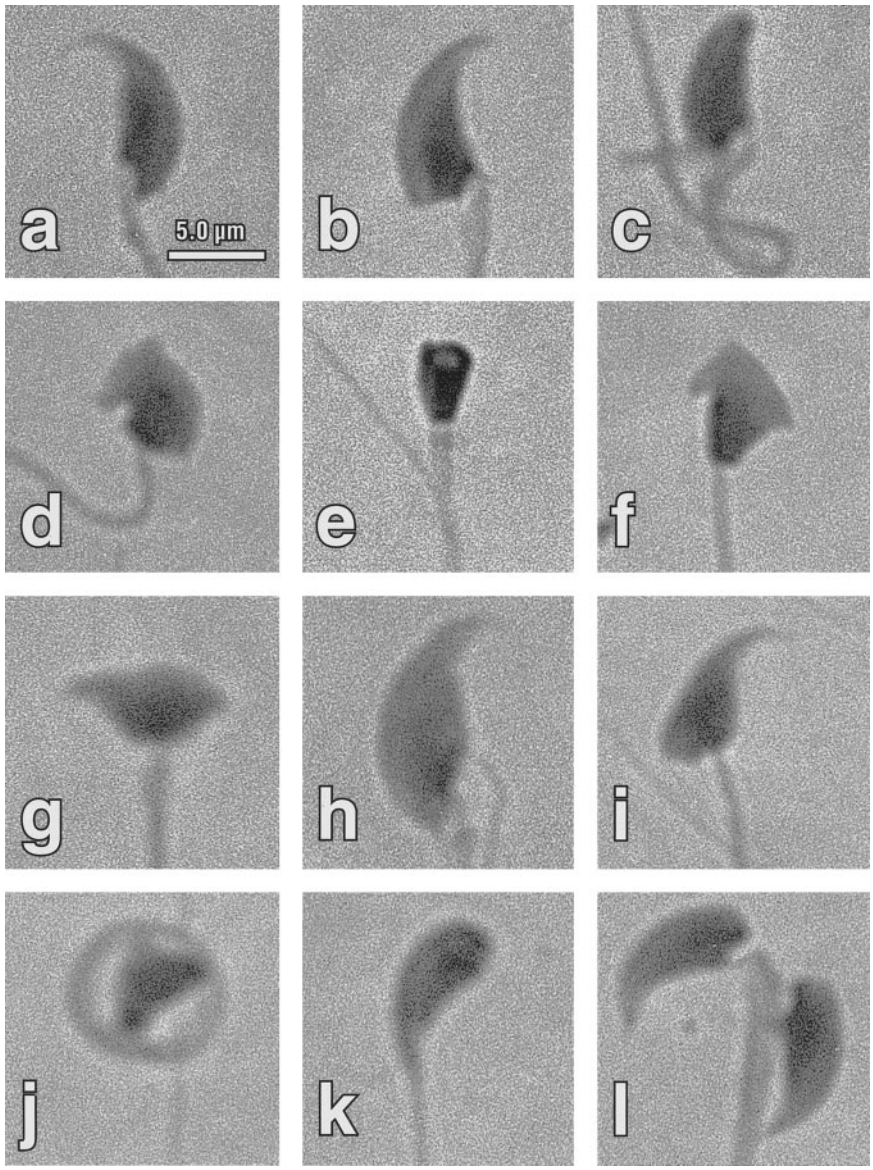

FIG. 2. Head morphology for CD1 mouse spermatozoa. Spermatozoa were categorized as normal (a) or abnormal (b–l). The abnormal group was further categorized into 11 subcategories: (b) amorphous; (c) acrosome; (d) truncated; (e) calyculate; (f) collapsed; g) triangular; (h) giant; (i) hammer; (j) tail; (k) inverted; and (l) double headed. Sperm were fixed and stained with hematoxylin. Magnification for all panels shown by 5-μm scale in a.

chosen for the postfertilization studies. The mean embryo cell number data was obtained by taking a random sample from those female mice that had produced greater than 10 two- to four-celled embryos.

The dose group consisting of 10 males was given a single dose of methamidophos (5.0 mg kg^{-1} body wt in 0.1 ml saline) and then each was mated for 7 consecutive weeks to one superovulated female per week. The control group consisting of 15 males was given an injection of 0.1 saline and also each was mated to one superovulated female per week for 7 consecutive weeks. This design permits the evaluation of progressively earlier stages of spermatogenic cells that were exposed to methamidophos; postexposure week 1, spermatozoa; postexposure weeks 2–3, spermatids; postexposure weeks 4–5, spermatocytes; postexposure weeks 6–7, intermediate and differentiating late Type A/Type B spermatogonia. An additional advantage of this design is that each postexposure week had its own concurrent control dose group.

CD1 female mice were housed under controlled lighting (14D:10L). Females were superovulated with 5 IU pregnant mare's serum gonadotropin (PMSG; Sigma) and given 5 IU human chorionic gonadotropin (hCG) 48 h post-PMSG to induce ovulation. Females were placed in cages one female per male immediately following hCG injection and left with males for 48 h post-hCG.

Embryos were flushed from excised oviducts at 50 h post-hCG (Warner *et al.*, 1991). Upon collection from the oviduct, the number of two- to four-celled embryos, the number of degenerating embryos, and the number of one-celled embryos or unfertilized eggs were recorded per male. The embryos were classified as follows: Normal healthy two- to four-celled embryos were those that contained well-developed, distinct blastomeres (Hogan *et al.*, 1986). For degenerating embryo and fertility data, embryos that had undergone fragmentation and/or that had dead blastomeres were classified as degenerate embryos at the time of recovery from the oviducts (Alikani *et al.*, 1999). One-celled embryos or unfertilized eggs were grouped together as one category because

TABLE 1

Postdose Cholinesterase Activity Levels per Animal and Mean Cholinesterase Activity Levels per Dose Group for the Final Range-Finding Assay (Study 3)

Methamidophos dose (mg kg^{-1} body wt)	Postdose cholinesterase activity per animal (μmol min^{-1} ml^{-1})	Mean cholinesterase activity per dose group (μmol min^{-1} ml^{-1})[a]
0.0	2.18	2.07 ± 0.08
	2.10	
	1.84	
	1.98	
	2.26	
0.5	2.30	2.01 ± 0.18
	2.10	
	1.41	
	2.20	
	2.10	
3.75	1.26	1.15 ± 0.11[b]
	1.22	
	0.78	
	1.37	
	1.12	
5.0	0.83	0.86 ± 0.16[b]
	1.10	
	0.43	
	1.13	
7.5	0.89	0.76 ± 0.06[b]
	0.74	
	0.78	
	0.56	
	0.82	

Note. Cholinesterase activity levels were determined on blood samples collected from the tail of male mice 24 h after methamidophos dosing.

[a] Values are means ± SEM.

[b] Significantly different from control (one-tailed Student's *t* test, $p < 0.025$).

they were not clearly distinguishable from one another at the time of embryo collection (50 h post-hCG) and were therefore not included in the total embryo counts. Females that did not respond normally to superovulation were excluded from this study (Hogan *et al.*, 1986). Therefore, only females producing greater than 10 embryos with two to four distinct blastomeres were counted as pregnant.

Two-celled embryos with symmetrical blastomeres were cultured in modified (Wiley *et al.*, 1986) T6 culture medium (Quinn *et al.*, 1982) for 48 h after embryo collection. Embryos were incubated in 0.5% sodium citrate and fixed onto glass slides using glacial acetic acid:ethanol (1:1) and their nuclei were stained in Giemsa for 20 min. The nuclei were counted as a measure of embryo cell number. Nondividing embryos numbers were determined but were eliminated from the statistical comparison of mean cell number per embryo. Nondividing embryos were those that did not cleave beyond the two- to four-cell stage while in culture for 48 h.

Statistics

Group effect trends were evaluated by linear regression analysis of the group means using INSTAT (Graphed Software, San Diego, CA) and CA-Cricket Graph III (Computer Associates International, Inc., Islandia, NY). Group means represent the overall group trends, while individual values are subject to variation due to both normal biological differences and potential responses to treatment. Unlike the individual values, the uncertainties associated with the mean values are expected to be normally distributed by the central limit theorem of statistics and they can be subjected to *t* test of the zero slope hypothesis to determine if the observed trends are statistically significant.

Other statistical analysis of data include the following: Parametric analysis of the sperm morphology data was done by a nested ANOVA (SuperANOVA, Abacus Concepts, Berkeley, CA). Kruskal–Wallis nonparametric ANOVA and a one-way ANOVA were analyzed by the statistical program INSTAT. Chi-square data were analyzed using the statistical program STATVIEW. All other data were analyzed by a one-tailed or a two-tailed Student's *t* test.

RESULTS

Range-Finding and Cholinesterase Activity

In Study 1, the group of four males that received a 15.0 mg kg^{-1} body wt dose died within 30 min of a single ip injection. All animals in the other two dose (0.5 and 2.5 mg kg^{-1} body wt) groups survived. Severe symptoms associated with neurological toxicity were not observed in doses of 2.5 mg kg^{-1} and lower. Mean cholinesterase activity was decreased for dose groups 2.5 mg kg^{-1} body wt (1.44 ± 0.16 μmol min^{-1} ml^{-1}) and 0.5 mg kg^{-1} body wt (2.04 ± 0.20 μmol min^{-1} ml^{-1}) compared to concurrent controls (2.5 ± 0.30 μmol min^{-1} ml^{-1}) and predose levels of dosed males (2.6 ± 0.14 μmol min^{-1} ml^{-1}). Since the 15.0 mg kg^{-1} body wt dose was lethal, a second study was done to examine the effects of 5.0 and 10.0 mg kg^{-1} body wt doses as well as a lower dose of 1.0 mg kg^{-1} body wt.

In Study 2, one of two males died within 30 min after injection of a 10 mg kg^{-1} body wt dose. The remaining male exhibited severe tremors, spasms, loss of balance, lacrimation, and fecal staining. These visible symptoms were extreme for 2 h and lessened after 5 h of injection. The males injected with a dose of 5.0 mg kg^{-1} per body wt also exhibited these same severe symptoms, but recovered from visible symptoms by 3 h after being treated. Animals that were dosed with 1.0 mg kg^{-1} body wt did not display any visible neurological responses. Cholinesterase activity levels were measured and mean cholinesterase activity levels were decreased for all dose groups, 1.0 mg kg^{-1} body wt (1.5 ± 0.2 μmol min^{-1} ml^{-1}), 5.0 mg kg^{-1} body wt (1.0 ± 0.2 μmol min^{-1} ml^{-1}), and 10.0 mg kg^{-1} body wt (0.6 μmol min^{-1} ml^{-1}) compared to the mean for all predose levels (2.6 ± 0.1 μmol min^{-1} ml^{-1}) of the dosed males and controls (2.5 ± 0.2 μmol min^{-1} ml^{-1}).

Study 3 utilized doses of 0.0, 0.5, 3.75, 5.0, and 7.5 mg kg^{-1} body wt and provided an MTD and observations on spermatid counts and sperm morphology. A new dose of 7.5 mg kg^{-1} body weight was added since the preliminary Studies 1 and 2 revealed that the MTD appeared to lie between 10 and 5 mg kg^{-1} body wt. This third study revealed that the 7.5 mg kg^{-1} body wt was the MTD for this study. This 7.5 mg kg^{-1} body wt dose was the highest dose that resulted in no deaths and no more than 10% loss of weight over a period of 4 weeks after administration of methamidophos (Generoso and Piergorsch,

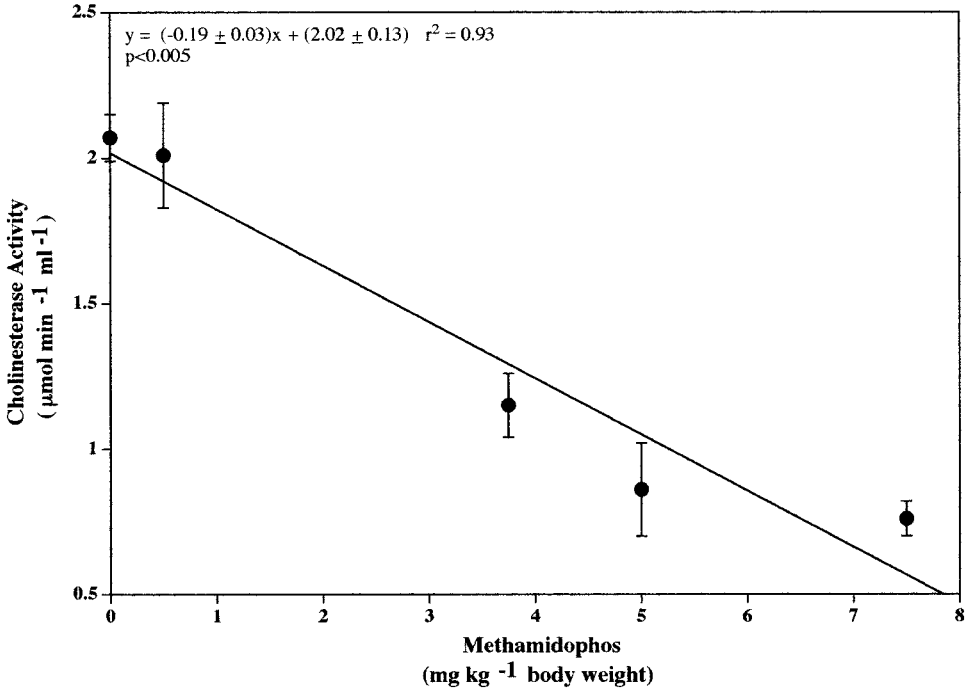

FIG. 3. Mean cholinesterase activity levels versus dose group. Data points (●) represent the mean cholinesterase activity levels (μmol min^{-1} ml^{-1}) per dose group measured 24 h after methamidophos dose. The slope of the line is significantly different from zero (t for zero correlation is 6.5, $p < 0.005$, one-tailed Student's t test). The bars represent the SEM.

1993). Males were weighed weekly for 4 weeks after dosage and none were found to exhibit significant loss of body weight (data not shown). Measurement of plasma cholinesterase activity revealed that control levels were significantly greater than any of the postexposure levels, except for the 0.5 mg kg^{-1} body wt dose group. Evidence of cholinesterase inhibitor exposure was confirmed by a dose–response in plasma cholinesterase activity in blood samples drawn 24 h after the methamidophos dose (Table 1 and Fig. 3). Regression analysis of the mean cholinesterase activity levels per group also revealed a significant dose–response effect (t for zero correlation = 6.5, $p < 0.005$, one-tailed Student's t test, Fig. 3). Animals that were dosed with 5.0 and 7.5 mg kg^{-1} body wt showed physical symptoms of anticholinesterase treatment, which included severe tremors, lacrimation, urine, and perianal staining for 2 to 4 h after dosing (Sheets *et al.*, 1997). Plasma cholinesterase activity varied but the levels in the higher dose ranges (3.75 mg kg^{-1} body wt and greater) exhibited a reduction in plasma cholinesterase activity.

TABLE 2
Mean Number of Spermatids per Gram Testes

Methamidophos dose (mg kg^{-1} body wt)	No. of animals	Mean number of spermatids per gram testes[a] ($\times 10^8$)[b]
0.0	5	1.30 ± 0.08
0.5	5	1.37 ± 0.06
3.75	5	1.57 ± 0.19
5.0	5	2.11 ± 0.24
7.5	6	1.64 ± 0.20

Note. Testes were homogenized and spermatids were counted on a hemocytometer as in Blazak *et al.* (1993).

[a] The mean number of spermatids per gram testes was not significantly different among the dose groups (two-tailed Student's t test, $p > 0.05$).

[b] Values are means ± SEM.

Spermatid Quantitation

The number of spermatid nuclei per gram testes was evaluated to provide some measure of the capacity of methamidophos to affect spermatogenesis. The mean number of spermatids per gram testes did not differ significantly among the dose groups compared to controls (two-tailed Student's t test, $p < 0.05$, Table 2).

Abnormal Sperm Morphology

Abnormal sperm morphology was evaluated to provide an indirect measure of the capacity of methamidophos to exert a potential effect on the genetic component of spermatogenesis. We observed a dose–response increase in the mean percent of abnormal sperm morphology per group, with increasing doses of methamidophos (Fig. 4). Regression analysis revealed a significant dose–response effect (t for zero correlation = 6.1, $p < 0.005$, one-tailed Student's t test; Fig. 4). This dose–

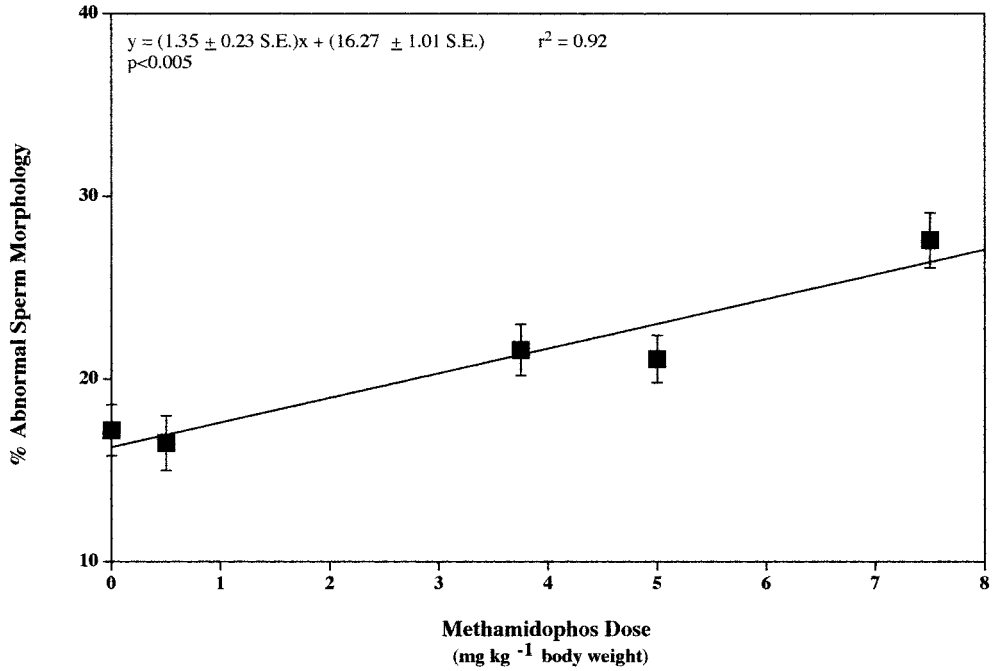

FIG. 4. Mean percents of abnormal morphology in males exposed to methamidophos. Data points (■) represent the mean percent abnormal sperm morphology per dose group from the following dose groups: 0.0, 0.5, 3.75, 5.0, and 7.5. The slope of the line is significantly differently from zero (t for zero correlation is 6.12, $p < 0.005$, one-tailed Student's t test). The bars represent the SEM.

response effect was further supported by a nested ANOVA ($p < 0.0001$, Table 3). Interanimal variation was also observed by a nested ANOVA ($p < 0.0001$).

Mean Cell Number per Embryo and Fertility

Mean cell number per embryo of preimplantation embryos after 48 h of culture was evaluated as a measure of transmitted effects from paternal exposure to methamidophos. This endpoint was selected because of its recognition as a stringent test of embryo vigor (Warner *et al.*, 1991). Mean cell number per embryo was evaluated after 48 h of culture from the two-cell stage to maximize any potential differences in cell proliferation rates between the dose and control groups of embryos (Warner *et al.*, 1991). There was significant decrease in mean cell number per embryo for the dose groups of embryos beginning with postexposure week 4 ($p < 0.03$, one-tailed Student's t test) and this decrease became exacerbated for postexposure week 5 ($p < 0.0001$, one-tailed Student's t test, Table 4). For postexposure week 6, this decrease was not evident, but the number of embryos that failed to cleave had increased significantly for this postexposure week (Table 5).

Beginning with postexposure week 4 there was also a significant increase in the proportion of degenerating embryos that became more pronounced with postexposure weeks 5 and 6 (Table 5). Data for the individual males were analyzed for interanimal variations by the Kruskal–Wallis nonparametric ANOVA ($p < 0.05$) and a one-way parametric ANOVA ($p < 0.05$). No significant difference between animals was found in these statistical tests.

The mean number of normal embryos produced by each male that mated can serve as a measure of overall fertility and this variable was also evaluated during the postexposure test period. There were no significant decreases or trends in this variable over the course of this test period except for postexposure week 6 (Table 5). Data for both mean number of embryos was analyzed for interanimal variations by the Kruskal–Wallis nonparametric ANOVA ($p < 0.05$) and a one-way parametric ANOVA ($p < 0.05$). No significant difference was found by either statistical test.

DISCUSSION

There were several new observations from this study on the germline effects of paternal methamidophos exposure in mice. First, there was a dose-dependent increase in abnormal sperm morphology. Second, beginning with postexposure week 4, we observed a significant decrease in embryo cell number of two-cell embryos after 48 h of culture that was accompanied by an increase in the proportion of degenerating embryos that were recovered per male. In addition, we determined that the MTD in the adult male for the CD1 strain of mouse was 7.5 mg kg^{-1} body wt and verified the presence of a physiological response to methamidophos exposure over a dose-range from 0.5 mg kg^{-1} body wt to the MTD.

TABLE 3
Percent Abnormal Sperm Morphology per Animal Exposed to Methamidophos

Methamidophos dose	Animal no.	Percent abnormal sperm morphology per animal[a]	Mean percent abnormal sperm morphology per dose group[b]
0.0	1	15.95 ± 1.3	17.2 ± 1.4
	2	15.30 ± 1.0	
	3	20.35 ± 2.0	
0.5	1	20.11 ± 2.2	16.5 ± 1.5
	2	14.95 ± 1.0	
	3	15.70 ± 1.4	
	4	17.56 ± 1.7	
	5	13.95 ± 1.3	
3.75	1	27.71 ± 0.6	21.6 ± 1.4[c]
	2	17.25 ± 1.5	
	3	34.95 ± 2.3	
	4	15.20 ± 1.0	
	5	13.20 ± 1.6	
5.0	1	18.42 ± 1.1	21.1 ± 1.3[c]
	2	18.55 ± 1.1	
	3	21.75 ± 1.6	
	4	27.15 ± 1.3	
	5	19.65 ± 1.2	
7.5	1	22.75 ± 1.9	27.6 ± 1.5[c]
	2	20.20 ± 1.1	
	3	27.70 ± 1.7	
	4	21.85 ± 0.9	
	5	58.40 ± 1.7	
	6	14.45 ± 1.4	

Note. Approximately 2000 sperm were counted per animal.
[a] Values are percents ± SEM.
[b] Values are means ± SEM.
[c] Significantly different from controls (Nested ANOVA, $p < 0.0001$).

Abnormal sperm morphology has been used previously as an endpoint for germline genetic effects from paternal exposures to a variety of toxicants (reviewed by Wyrobek *et al.*, 1983). The published data on chemical mutagenic responses of the mouse paternal germline indicate that the fourth week postexposure is when the most significant increases in abnormal sperm morphology are observed (Wyrobek and Bruce, 1975). This provided the rationale in our study for performing the evaluation of sperm morphology 4 weeks after methamidophos exposure.

Therefore, two sperm parameters were used to test for physiological effects in the testes after methamidophos exposure, spermatid counts to assess for disruption of spermatogenesis and abnormal sperm morphology as a indirect indicator of genetic damage. The spermatid counts were unremarkable, while the abnormal sperm morphology resulted in a small but statistically significant increase for the higher doses. Small but significant increases in the percentage of abnormal sperm morphology after organophosphate exposure have been previously reported in mice ranging from 4–12% (Jayashree *et al.*, 1994; Mathew *et al.*, 1992; Behera and Bhunya, 1989) and are correlated to doses resulting in chromosomal aberrations and micronuclei in bone marrow cells (Jayashree *et al.*, 1994; Behera and Bhunya, 1989). Therefore, the increases in abnormal sperm morphology of 3.9–10% observed for the doses of 3.75, 5.0, and 7.5 mg kg^{-1} body wt in this study are within these previously published ranges. It may be that the mutagenic capacity of organophosphates does not always correlate with induction of large numbers of abnormal sperm as previously seen with other toxicants but functions as a useful biomarker of germline effects (Wyrobek and Bruce, 1975; Wyrobek *et al.*, 1983). In this study, we observed a small but statistically significant increase in abnormal sperm morphology together with poor preimplantation development and fertility collectively suggesting the mutagenic potential of the organophosphate.

While our observations of transmitted embryonic effects from paternal methamidophos exposure are new, embryonic/developmental effects have been reported for maternal meth-

TABLE 4
Mean Cell Numbers per Embryo at 48 h in Culture Conceived after Paternal Exposure to Methamidophos at 5.0 mg kg^{-1} body wt

Postexposure week	3	4	5	6
Mean cell numbers per embryo at 48 h in culture[a]				
Control	23.4 ± 0.6 (12)	44.1 ± 0.4 (34)	30.9 ± 0.2 (42)	22.4 ± 0.5 (23)
Dose	21.7 ± 0.3 (14)	36.5 ± 0.7[b] (25)	25.7 ± 0.2[c] (39)	22.2 ± 0.7 (11)
No. of nondividing embryos/total (%)[d]				
Control	0/12 (0)	0/36 (0)	0/42 (0)	1/26 (3.8)
Dose	0/14 (0)	1/25 (4)	2/41 (4.9)	4/15 (26.6)

Note. Embryos were collected at the two-cell stage, cultured 48 h *in vitro* to morulae, fixed, and stained in Giemsa. The number of nuclei were counted per embryo.
[a] Values are means ± SEM with no. of embryos in parentheses.
[b] Significantly different from control (one-tailed Student's *t* test, $p < 0.03$).
[c] Significantly different from control (one-tailed Student's *t* test, $p < 0.0001$).
[d] Nondividing embryos were those with 10 or fewer blastomeres after 48 h in culture.

TABLE 5
Comparisons of the Mean Numbers of Two- to Four-Cell Embryos per Dose Group and the Percent Degenerated Embryos Produced by Males Exposed to Methamidophos and Their Concurrent Controls

Postexposure week	Total no. of pregnant females[a]	Total no. of embryos[b]	Total no. 2–4-cell embryos[c]	Mean No. 2–4-cell embryos per male[d]	Total no. of degenerated embryos[e]	Percent degenerated embryos[f]	*P* value for percent degenerated embryos[g]
Week 1							
Control	10	394	341	34.1 ± 3.9	53	15.5	
Dose	5	214	179	30.2 ± 7.4	35	16.4	0.3312
Week 2							
Control	10	334	285	28.5 ± 3.3	49	14.6	
Dose	7	215	194	27.7 ± 3.9	21	9.7	0.0927
Week 3							
Control	10	203	185	18.5 ± 1.8	18	8.9	
Dose	6	106	91	15.2 ± 1.5	15	14.2	0.1534
Week 4							
Control	9	324	300	33.3 ± 5.1	24	7.4	
Dose	6	220	191	31.8 ± 6.0	29	13.2	0.0260
Week 5							
Control	6	159	137	22.8 ± 2.7	22	13.8	
Dose	7	213	164	23.4 ± 3.3	49	23.0	0.0260
Week 6							
Control	9	390	358	39.8 ± 5.2	32	8.2	
Dose	5	141	112	22.4 ± 6.3[h]	29	21.0	<0.0001

[a] Only females with greater than 10 embryos with two- to four-celled blastomeres at the time of collection from the oviduct (50 h post-hCG) were considered pregnant.
[b] Total number of embryos includes two- to four-celled and degenerated embryos, one-celled embryos were not included as described under Methods.
[c] Total number of two- to four-celled embryos collected 50 h after post-hCG.
[d] Variation among the animals was not significant by the Kruskal–Wallis nonparametric test ($p > 0.05$) or a one-way parametric ANOVA ($p > 0.05$). Values are means ± SEM.
[e] Embryos that were fragmented and/or that had degenerate blastomeres at the time of collection from the oviducts were classified as degenerated embryos.
[f] Variation among the animals for the number of degenerated embryos per animal was not significant by the Kruskal–Wallis nonparametric test ($p > 0.05$) or one-way parametric ANOVA ($p > 0.05$).
[g] Chi-square test performed on values of total number of embryos and total number of degenerating embryos.
[h] Significantly different from postexposure week 6 concurrent control (one-tailed Student's *t* test, $p = 0.031$). A *p* value equal to 0.031 suggests a 17% probability that this is a random observation.

amidophos exposure (Hanafy *et al.*, 1986). Pregnant rats that were given methamidophos orally exhibited several indicators of adverse developmental effects, including decreases in implantation sites and total number of fetuses (live and dead) and increases in several teratological endpoints (Hanafy *et al.*, 1986). For maternal exposures, however, there are confounding issues of maternal somatic effects during oogenesis and gestation that make it difficult to evaluate the maternal germline for the potential of transmitted effects. One such confounder is the potential for mitochondrial genetic alterations arising within the oocyte.

We selected a preimplantation endpoint for embryo vigor, embryonic cell number (Warner *et al.*, 1991; Kelly and Roussant, 1976), as a starting point for evaluating the potential for developmental (postfertilization) effects following paternal germline methamidophos exposure. Mean cell number per embryo at 48 h in culture was significantly lower beginning with embryos conceived 4 weeks after paternal methamidophos exposure. This observation correlates with the significant increase in abnormal sperm morphology that was also apparent 4 weeks after paternal methamidophos exposure. This trend for increasing adverse effects beginning with postexposure week 4 was also supported by the significant rise in proportion of degenerating embryos that were recovered for postexposure weeks 4, 5, and 6 (Table 5). Since the adverse effect on embryonic cell proliferation became progressively more severe for embryos conceived 4, 5, and 6 weeks after paternal methamidophos exposure, we speculate that progressively more adverse postnatal developmental effects might exhibit a similar time course. The embryos that were conceived 6 weeks after paternal methamidophos exposure also exhibited a notable increase in cleavage arrests (Table 4), which is consistent with the idea that this postexposure week might be particularly prone to exhibiting adverse developmental effects following implantation.

Whether our observed effects on sperm morphology and preimplantation embryonic cell proliferation stem from "genetic" damage is an important question. Favoring the hypoth-

esis for genetic damage are the reported increases in sperm aneuploidy following occupational exposures in humans (Padungtod *et al.*, 1999) and increases in heritable cell proliferation effects for offspring that were conceived 6 weeks after paternal irradiation of mice with ^{137}Cs γ-rays (Warner *et al.*, 1991; Wiley *et al.*, 1997). Neither our observations nor those of others allow identification of the mechanism by which methamidophos might "damage" DNA. While some organophosphates are known alkylating agents, the bulk of the published data is more consistent with most organophosphate pesticides, including methamidophos, lacking action as alkylating agents, except at very high doses (Zayed and Mahdi, 1987). Phosphorylation is the more commonly seen action of organophosphate pesticides (Bedford and Robinson, 1972), which leads to lowered cholinesterase activity in red blood cells, in plasma, and in the brain (Zayed *et al.*, 1984; Sheets *et al.*, 1997). We speculate that methamidophos might contribute to "genetic" damage of sperm chromatin by promoting the phosphorylation of structural and/or enzymatic components of the mitotic spindle to result in mitotic/meiotic errors. This idea has been invoked previously for the sperm centrosome, which plays a key role in the establishment and function of the first zygotic mitotic spindle (Navara *et al.*, 1997).

In conclusion, we observed that an acute paternal exposure to methamidophos produced a dose-dependent increase in abnormal sperm morphology evaluated 4 weeks after exposure. In addition, beginning with postexposure week 4, there was an adverse effect on embryonic cell proliferation in cultured preimplantation embryos and significant increases in the proportion of degenerate embryos recovered per male. Work is underway to test hypotheses that these effects correlate with increases in chromosomal aberrations and/or additional forms of genetic damage in the male germline.

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