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Effects of separate and combined exposure to the pesticides methamidophos and chlorothalonil on the development of suckling rats

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Abstract

It has been suggested that, in some cases, exposure to environmental contaminants affects children more profoundly than adults. It is important to evaluate adverse health outcomes in children, a population susceptible to toxic chemicals and mixtures. We examined the effects that maternal exposure to two pesticides had on maturational aspects of offspring development during the nursing period. Nursing female rats were exposed to 1–4 mg/kg of intraperitoneal methamidophos, 200–800 mg/kg of chlorothalonil, or both. The higher doses of methamidophos affected pup viability by day 21 of life. Both pesticides, alone or together, affected body weight gain of dams and offspring. Developmental milestones evaluated in the pups were incisor eruption, ear unfolding, eye opening and testis descent. Although no clear dose–response relationship was established between these milestones and exposure to methamidophos or chlorothalonil, incisor eruption was accelerated in many groups, and the majority of rat offspring exposed to methamidophos presented later ear unfolding and eye opening than did the control group offspring. Sexual maturation (testis descent) was significantly delayed in some groups. For dams and pups alike, simultaneous exposure to both pesticides was not found to have a greater toxic effect than that resulting from exposure to only one of the two. Taken together, these results demonstrate exposure-related influences on several developmental measures. Detection of more subtle effects may be improved through the use of the developmental temporal response protocols utilized in this study.

Keywords: Methamidophos; Chlorothalonil; Suckling rats; Pesticide mixtures; Physical milestones; Perinatal exposure

Introduction

Poisoning in newborns has been associated with maternal exposure to pesticides at the place of residence or in the workplace (Whyatt et al., 2002; Cerrillo et al., 2005; Ribas-Fitó et al., 2005). Multiple pesticides can be present simultaneously in breast milk. The quantity of pesticide that is passed to the infant via breast milk is influenced by many variables (Weiss et al., 2004).

Age-dependent variations in susceptibility appear to depend on the chemical of concern, the effect that is evaluated, the level/duration of exposure and the developmental period during which the exposure occurred (Scheuplein et al., 2002). It is also noteworthy that infants may be more susceptible to neurobehavioral alterations, which may not be apparent until later in life (Moser et al., 2001; Scheuplein et al., 2002; Costa et al.,

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2004). For example, in experimental models, developing animals have been shown to be more susceptible than adult animals to the acute toxicity of the organophosphate (OP) pesticide chlorpyrifos, which can cause neurobehavioral abnormalities (Richardson and Chambers, 2005).

When two or more chemicals are applied simultaneously to a living system or unit, the combined effect can modify the individual toxic effect, since they might have cellular targets or metabolic pathways in common.

Despite the fact that almost all chemical exposure of humans is to mixtures, and that such mixed exposure occurs in the context of numerous other risk modifiers, the current understanding of human health risks is based almost entirely on the evaluation of chemicals studied in isolation. Under realistic environmental conditions, concomitant or sequential exposure to pesticide mixtures dictates the need for exposure assessment, hazard identification and risk assessment (Cory-Slechta, 2005).

Attempts to deal with the problem of chemical mixtures have largely been restricted to classes of chemicals that are structurally related. While an intraclass focus may be a logical starting point for evaluating the toxicity of chemical mixtures, it encompasses only a small fraction of the aggregate or overall exposure problem. The breadth of classes and types of chemicals to which humans are exposed are not structurally-related chemicals (Cory-Slechta, 2005). Better understanding of the patterns of exposure and the underlying variability within the human population, as well as the links between the animal toxicology data and human health effects, will improve the evaluation of risks to human health posed by pesticides mixtures (Lima, 2003; Mumtaz et al., 2004).

The OP insecticide methamidophos and the fungicide chlorothalonil are commonly used in agriculture and are frequently sprayed simultaneously on various crops, including tomatoes (Castro et al., 1999). The neurotoxic potential of OP insecticides has been described by various authors (Farahat et al., 2003; Smulders et al., 2004). These insecticides exert acute toxicity primarily through persistent inhibition of acetylcholinesterase (AChE) at cholinergic junctions within the central and peripheral nervous systems. Although AChE is the clinically significant target of OP exposure, other proteins also form covalent bonds with OP insecticides, depending on the chemical properties of the insecticide (Peeples et al., 2005). However, the toxicological relevance of the inhibition of these secondary targets is not yet understood. In addition, there are examples of signs of toxicity unaccompanied by AChE inhibition. In experimental animal models, doses that are too low to produce cholinergic signs have been shown to produce a variety of effects, ranging from enhanced maze learning to slowed nerve conduction (Peeples et al., 2005). These toxicological observations suggest that OP insecticides have biological effects other than their cholinesterase-inhibitory properties. The use of other end measures could increase the ability to identify toxic responses to chemicals polluting the workplace and the general environment.

In contrast to the relatively extensive knowledge regarding OP compounds, there are no well-defined biomarkers of the effects of chlorothalonil. Despite the fact that mixtures (including chlorothalonil) simulating groundwater contamination have been shown to reduce blastocyst development and the mean number of blastocysts per embryo (Greenlee et al., 2004), chlorothalonil toxicity has been evaluated only in adults.

Since chlorothalonil has the potential to promote hepatic and renal damage (Suzuki et al., 2004) and consequently alter drug metabolism and excretion, it might be expected that the toxicity of methamidophos would be altered when there is concurrent exposure to the two pesticides, especially during lactation.

Owing to the present lack of information concerning the effects of mixtures of toxicants on postnatal development, we studied the effects that exposing dams to chlorothalonil and methamidophos mixtures had on suckling rats. To that end, we constructed dose–response curves for one compound in the presence of a range of doses of the other.

Since it has been established that the first three weeks postpartum include a period of brain growth, during which the most rapid development of cholinergic neurons occurs in the rat brain (Tang et al., 2003), we studied the effects that lactational exposure to various mixtures of methamidophos and chlorothalonil had on suckling rats between postnatal days (PND) 1 and 21. The dams were exposed intraperitoneally during lactation, which results in first-pass metabolism in the liver. There is some evidence that methamidophos, acting directly or as a metabolite, is a potent liver carboxyamidase inhibitor in mammals (Mahajna et al., 1997). Chlorothalonil has been shown to induce lipid peroxidation in rat hepatocytes in vitro and has been classified as a human carcinogen of intermediate potency (Lodovici et al., 1997).

Materials and methods

Animals

Ninety-day-old Wistar male and nulliparous female rats from our own colony, weighing 250 ± 30 g, were used. Females were housed individually in polycarbonate cages with hardwood chip bedding and were given free access to tap water and food. Within the facilities, the environment was controlled (room temperature:

22±2 °C; humidity: 70%), and a 12 h light/dark cycle was maintained. The animals were fed a commercially prepared laboratory diet (Purina Lab Chow). The P-generation breeders of our own colony come from Cemib–Unicamp facilities (Campinas, Brazil), which is recognized by the International Council for Laboratory Animal Science. The general health status of our colonies is controlled using standard operating procedures (ISO 9001:2000).

Vaginal smears were examined daily for 12 days prior to mating and pesticide exposure. The estrous cycle phase of each female was observed, and any females presenting abnormalities were excluded. Males, previously determined to be fertile, were housed overnight with females. The presence of a vaginal plug or spermatozoa in the vaginal smears of females on the following morning was considered evidence of mating, and that day was designated gestational day 0.

Pesticides

The commercial pesticides tested were methamidophos (Tamaron®, 600 kg/l; Bayer CropScience, Alemania, Brazil) and chlorothalonil (Vanox®, 750 g/kg; Syngenta, São Paulo, Brazil). Pesticides were suspended in 0.9% saline solution, to which two drops of Tween 80 had been added. Controls were injected with saline solution plus Tween 80 alone. The volume of Tween 80 was taken into account in the calculation of the final volume of the control or pesticide suspension solutions. Doses are expressed in mg/kg of the active ingredient (methamidophos or chlorothalonil) of the products (Tamaron® or Vanox®, respectively).

Procedures

Dosing

The females were divided, 10 dams per group, into nine groups: eight treatment groups and one control group. The sperm-positive females were randomly assigned to control or treatment groups, which received saline solution or pesticide suspension solution, respectively. Treatment group females were injected with methamidophos (MT 1 = 1 mg/kg; MT 2 = 2 mg/kg; MT 4 = 4 mg/kg), chlorothalonil (CR 200 = 200 mg/kg; CR 400 = 400 mg/kg; CR 800 = 800 mg/kg) or both (MT 2 + CR 800; MT 4 + CR 800). The dams were exposed intraperitoneally during lactation (between PND 1 and 21).

To verify whether there was a dose–response relationship for the biomarkers measured, we initially determined the sequential doses that might or might not cause clinical signs of poisoning, such as salivation, dyspnea, tremors and seizures. Methamidophos lowest dose did not promote evident signs of toxicity and the highest dose caused some weight reduction in the dams and resulted in pup death at the end of the exposure period.

Since there were no signs of toxicity promoted by chlorothalonil exposure, only the highest one was tested combined to methamidophos.

Pup viability and weight

After mating, pregnant females were weighed and examined periodically for signs of toxicity or any other problems during pregnancy. Each presumed-pregnant female was checked twice daily (9:00 and 17:00 h) for completion of, or difficulties in, parturition until the completion of delivery. The day of parturition was defined as PND 1. However, for litters born after 17:00 h, the following day was considered PND 1. After parturition, the neonates were monitored for mortality and signs of toxicity. Growth and development were evaluated daily. Viability at birth and weaning were calculated, respectively, as (a) the total number of stillbirths and the number of neonatal deaths within 24 h and (b) the total number of weaned pups and the number of pups born alive. The results are expressed as percentages. On PND 21, pups were weaned, separated and housed, five to a cage, by sex based on anogenital distance.

Offspring body weights were recorded (in grams) on PND 7, 14 and 21 for all pups. The pups were weighed on PND 1 as a developmental control. Body weights were recorded during nursing. For each litter, the mean was assumed to be the litter weight, and each litter was considered an experimental unit.

Milestone development

The pups were removed from the nest with a paper towel and placed on a heating pad before and after testing. Physical development was evaluated, litter by litter, by recording the PND on which each of the following milestones appeared: pinna detachment (unfolding of the external ear); fur development; incisor tooth eruption; ear unfolding; eye opening; and testis descent (descent of the testicles into the scrotum). The eye opening and ear unfolding parameters were considered positive only when the eye/ear had opened/unfolded completely. Pups were examined daily to determine the PND on which the upper and lower incisors erupted (positivity for this parameter recorded only when all incisors had erupted). As a basis for postnatal studies, the time points at which physical milestones appeared were recorded and compared with the normal physical development of the species and strain used (Alder, 1983). The neurodevelopmental milestones were compared to literature and laboratory

historical data since similar experiments are performed repeatedly in our laboratory.

The days required for the appearance of these milestones were recorded until all pups in the litter were positive for all parameters. The frequency of animals presenting positivity for the parameters tested was recorded daily throughout the observational period. Litters were evaluated in random order.

Statistical procedures

The percentage variations in reproductive performance were analyzed using the chi-square test, and differences in body weight among groups were evaluated by ANOVA with repeated measures on the time factor (Morrison, 1976) using the general linear model procedure in the SAS program (SAS, 1988). For the analysis of significant deviations in the developmental milestones, survival (lifetime) data analysis was used to evaluate the cumulative frequency of the appearance of each parameter in relation to animal age (Cox and Oakes, 1984). The level of statistical significance was set at $p<0.05$.

Results

Reproductive performance and pup viability

There were no differences among the groups in terms of fertility or of the number of viable pups at birth. However, pup viability on PND 21 was affected by the highest dose of methamidophos in the MT 4 and MT 4 + CR 800 groups (Table 1).

Table 1. Pup viability after maternal exposure ($n = 10$) to methamidophos and/or chlorothalonil, i.p. during PND 1–21

Groups	Viability (%) of pups at	
	Birth	Weaning
Control	100 (93/93)	100 (93/93)
MT 1	100 (106/106)	100 (106/106)
MT 2	99 (91/91)	100 (91/91)
MT 4	99 (93/94)	91 (85/93)*
CR 200	100 (81/81)	97 (78/81)
CR 400	100 (98/98)	100 (98/98)
CR 800	100 (90/90)	100 (90/90)
MT 2 + CR 800	100 (98/98)	96 (94/98)
MT 4 + CR 800	97 (88/91)	92 (81/88)*

The number of viable pups at the end of lactation on the 21st day of life was determined (χ^2 test, $^*p<0.05$). Viabilities at birth and weaning were defined, respectively, as the ratio between alive-born pups/pups born and weaned-pups/alive-born pups. Data were expressed as percentage.

Significant differences between the control group and the treatment groups were observed in relation to body weight. There were transient effects on body weight in MT 4, MT 2 + CR 800 and MT 4 + CR 800 group dams. Significant decreases in dam body weight were found in the MT 4 group on PND 7 ($F = 6.73$; $p<0.01$) and PND 14 ($F = 5.89$; $p<0.01$), which recovered by PND 21. On PND 14, dam body weight was also significantly lower in the MT 2 + CR 800 group ($F = 3.79$; $p<0.05$) and MT 4 + CR 800 group ($F = 8.75$; $p<0.004$). In addition, there was a statistically significant difference between the control and CR 400 groups (higher in the latter) on PND 21 ($F = 4.31$; $p<0.04$) (Table 2).

A statistically significant decrease in pup body weight was observed in the MT 2 group on PND 7 ($F = 7.29$; $p<0.008$) and in the MT 4 group on PND 7 ($F = 4.93$; $p<0.03$) and PND 14 ($F = 6.64$; $p<0.01$). Pup body weights were also significantly decreased on PND 21 in the MT 2 + CR 800 group ($F = 4.45$; $p<0.03$). However, there was a statistically significant increase in pup body weight in the CR 200 group on PND 7 ($F = 3.84$; $p<0.05$) and PND 21 ($F = 4.93$; $p<0.03$) (Table 3).

Milestone development

Incisor eruption, ear unfolding, eye opening and testis descent were evaluated as parameters of physical development. The results were analyzed by survival analysis in order to determine whether there was significant delay or acceleration of the developmental stages. Table 4 shows the percentage of pups not presenting the milestones evaluated on the day of examination. Statistical differences are given for the end of the observational period.

There were differences between the treated groups and the control group in the percentage of animals presenting each of the parameters on a certain day of life. However, in the absence of a dose–response relationship, the appearance of some milestones was affected, as described below, in offspring exposed to methamidophos, chlorothalonil or both.

Incisor eruption was accelerated in many of the groups: CR 200; CR 400; CR 800; MT 2 + CR 800; and MT 4 + CR 800 ($p<0.05$), but delayed in MT 2 group.

Ear unfolding and eye opening occurred later in the majority of pups exposed to methamidophos than in the control group. Ear unfolding was also significantly delayed in the MT 2, MT 4, CR 200, CR 800, MT 2 + CR 800 and MT 4 + CR 800 groups. In contrast, ear unfolding was significantly accelerated in the CR 400 group. Eye opening was significantly retarded in the MT 1, MT 2, MT 4 and MT 2 + CR 800 groups.

The physical parameter of sexual maturation (testis descent) was significantly delayed in the MT 1, MT 2,

Table 2. Weight of dams exposed to methamidophos and/or chlorothalonil, i.p. during lactational days 1, 7, 14 and 21($n = 10$)

Groups	Control	MT 1	MT2	MT 4	CR 200	CR 400	CR 800	MT 2 + CR 800	MT 4 + CR 800
PND 1	248.0 ± 17.2	237.3 ± 11.6	251.5 ± 26.5	242.4 ± 15.1	237.0 ± 12.5	256.3 ± 11.9	257.7 ± 9.6	235.1 ± 6.2	237.0 ± 12.1
PND 7	265.3 ± 10.5	256.0 ± 12.1	253.4 ± 36.3	243.7 ± 19.2*	257.6 ± 19.1	278.7 ± 17.6	278.5 ± 18.6	253.6 ± 5.3	255.0 ± 11.5
PND 14	289.2 ± 11.4	283.8 ± 16.6	281.2 ± 27.7	270.5 ± 23.5*	280.9 ± 11.2	301.2 ± 16.4	294.8 ± 19.0	274.2 ± 8.3*	266.4 ± 11.2*
PND 21	285.7 ± 11.4	283.8 ± 30.2	281.2 ± 21.6	287.0 ± 25.5	291.0 ± 15.2	303.9 ± 13.7*	290.4 ± 21.2	294.8 ± 13.4	271.0 ± 15.9

Data expressed as mean ± SD (grams), analysis of variance, * among groups, $p < 0.05$.

Table 3. Weight of pups exposed to methamidophos and/or chlorothalonil, i.p. during PND 1–21 at postnatal days 1, 7, 14 and 21 ($n = 10$)

Groups	Control	MT 1	MT2	MT 4	CR 200	CR 400	CR 800	MT 2 + CR 800	MT 4 + CR 800
PND 1	6.1 ± 1.0	6.4 ± 0.5	5.6 ± 1.0	6.3 ± 0.5	6.7 ± 0.7	6.8 ± 0.5	6.6 ± 0.5	5.8 ± 0.5	6.4 ± 0.4
PND 7	11.1 ± 1.7	11.6 ± 1.4	9.2 ± 1.4*	9.5 ± 1.6*	12.5 ± 2.1*	12.2 ± 1.2	11.9 ± 1.6	10.9 ± 1.2	11.1 ± 0.9
PND 14	20.5 ± 3.1	21.5 ± 2.7	17.7 ± 3.2	16.9 ± 3.5*	24.0 ± 4.0	22.2 ± 2.2	20.9 ± 3.0	18.5 ± 1.5	19.6 ± 2.4
PND 21	32.1 ± 4.5	32.4 ± 4.9	28.4 ± 4.3	27.8 ± 5.3	37.3 ± 6.9*	34.7 ± 4.4	33.1 ± 5.3	27.6 ± 2.3*	31.4 ± 4.0

Data were expressed as mean ± SD (grams), analysis of variance, * among groups, $p < 0.05$.

CR 200 and CR 800 groups in comparison with the control group, whereas such delays were not significant in the other groups tested ($p > 0.05$).

Discussion

The rat dam, as the primary source of nutrition, grooming and warmth required for survival, influences the development of major regulatory systems underlying behavior and physiology in the rat neonate (Huot et al., 2004). Although exposure to the highest dose of methamidophos resulted in lower body weights in dams and increased mortality in pups, we did not observe any overt symptom of cholinergic toxicity, such as tremors or convulsions. The highest dose of methamidophos also diminished the viability of pups at weaning, proving to be toxic both in isolation and in combination with chlorothalonil. The lower body weights among pups exposed to methamidophos could affect their development and retard growth. However, although there was no dose–response or temporal relationship, exposure to chlorothalonil, with the exception of the highest dose, promoted an increase in pup body weight. This finding is likely attributable to the body weights of the corresponding dams.

The effects of chlorothalonil on the appearance of developmental milestones in rat pups appear to indicate that chlorothalonil protects against methamidophos toxicity. However, Lodovici et al. (1997) purported that the toxicity of the low doses of pesticide mixtures present in foods could be reduced by eliminating chlorothalonil from such mixtures.

In view of these facts, we studied the postnatal effects of pesticide exposure on rat pup development. The delay in testis descent observed in the present study might indicate pesticide interaction with hormone homeostasis, since some pesticides can adversely affect reproduction and development. Such effects involve multiple pathways within the reproductive tract and hypothalamic–pituitary–gonadal axis, consequently promoting endocrine-disrupting effects (Dutta and Meijer, 2003; Cummings and Kavlock, 2004). These findings serve as a warning that pesticides can induce perinatal endocrine disruption after suckling exposure. Future biochemical studies should directly address these potential effects in order to test this hypothesis.

The data reported in this study show that there is not a dose–response relationship between concomitant lactational exposure to the two pesticides evaluated and the maturation process, as evaluated by observing milestones of physical development. In addition, for some of the pup maturation parameters tested, the toxic exposure levels for the two chemicals in the mixture were found to be equivalent to that of each chemical in isolation. Nevertheless, few statistically significant alterations were observed. There was a general delay in the appearance of developmental milestones, with exception of incisor eruption, which actually occurred earlier in many groups. Therefore, our results suggest that the pesticide combination tested does not constitute an increased hazard in comparison with isolated exposure to methamidophos or chlorothalonil.

Although it was difficult to detect a consistent effect of increasing doses of pesticides when given during lactation, nonlinear dose–responses are not uncommon and actually seem reasonable in light of the complexity of the systems that pesticides target. The absence of a dose–response relationship might result from the reactions of a complex biological system to a toxicant, since not all brain regions develop at the same rate (Costa et al., 2004).

Table 4. Milestone development of pups whose dams were exposed to methamidophos and/or chlorothalonil i.p. during lactation (PND 1–21)

Group	% Animal showing landmark on PND								
	Incisor eruption								
	4	5	6	7	8	9			
Control	0	42	85	100					
MT 1	0	40	92	100					
MT 2	0	47	67	87	97	100*			
MT 4	0	27	88	100					
CR 200	0	9	90	100*					
CR 400	0	51	97	100*					
CR 800	0	68	98	100*					
MT 2+CR 800	0	77	100*						
MT 4+CR 800	0	55	91	100*					
	Ear opening								
	10	11	12	13	14	15	16	17	18
Control	0	0	0	18	100				
MT 1	0	0	0	38	77	100			
MT 2	0	0	0	18	54	80	89	99	100*
MT 4	0	0	4	22	72	83	95	98	100*
CR 200	0	0	5	41	56	100*			
CR 400	0	0	15	88	100*				
CR 800	0	0	27	84	99	100*			
MT 2+CR 800	0	6	11	11	47	84	98	100*	
MT 4+ CR 800	0	1	14	39	64	100*			
	Eye opening								
	12	13	14	15	16	17	18	19	20
Control	0	0	0	20	61	94	100		
MT 1	0	0	1	10	38	73	100*		
MT 2	0	0	0	1	20	52	80	97	100*
MT 4	0	0	0	3	8	23	72	97	100*
CR 200	0	0	15	30	58	83	100		
CR 400	0	0	4	26	78	86	100		
CR 800	0	1	4	27	64	92	100		
MT 2+CR 800	0	0	5	19	55	73	97	100*	
MT 4+CR 800	0	0	1	10	64	82	94	100	
	Testes descent								
	18	19	20	21	22	23			
Control	0	47	67	100					
MT 1	0	0	0	80	100*				
MT 2	0	0	22	84	97	100*			
MT 4	0	26	62	88	100				
CR 200	0	0	7	95	100*				
CR 400	0	0	0	78	98	100			
CR 800	0	0	29	94	98	100*			
MT 2+CR 800	0	0	5	74	100				
MT 4+CR 800	0	0	45	80	93	100			

All pups of each litter were evaluated. The data were analyzed by survival data analysis (log-rank) as percentage of animals in relation to days of life till the end of the observational period, $^{*}p<0.05$.

Some general issues related to developmental testing strategies are raised by the results of our experiments. It is possible that the experimental protocol design, as currently used to identify physical milestones, lacks the detainment form to detect the physical development associations suspected. Such associations might be better determined by using shorter observation intervals, since the development of physical milestones appears to be very sensitive to the effects of pesticides (Castro et al., 2000; Bushnell et al., 2002).

Taking into account the complexity of animal and human physiology, experimental tests and epidemiological evaluations should be used in combination in order to characterize the potential toxic effects of single pesticides and pesticide mixtures.

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