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POSTNATAL DEVELOPMENT AND FERTILITY OF OFFSPRING FROM MICE EXPOSED TO TRIPHENYLTIN (FENTIN) HYDROXIDE DURING PREGNANCY AND LACTATION

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Fentin or triphenylthin (TPT) is an organotin compound (OTC) widely used as an agricultural fungicide and miticide. It is well known that TPT exerts adverse effects on the reproductive and immune systems and may disrupt the endocrine system, raising concerns regarding the risks posed by exposure to this metal on environmental and human health. In this study the effects of maternal exposure to TPT at doses of control (0), 1.875, 3.75, or 7.5 mg/kg body weight/d, po, were examined during gestation and lactation on offspring growth, organ weights, and fertility. Except for a significant liver enlargement at the highest dose, TPT produced no maternal toxicity. Increased neonatal mortality (death of 3 entire litters from a total of 18 treated litters) was noted at 7.5 mg/kg. Pup body weight at birth was significantly reduced at all dose levels, but no marked weight loss was found on postnatal day (PND) 5 and thereafter. Offspring maturation (ear unfolding, incisor eruption, vagina opening, and testes descent) and fertility in adulthood were not significantly affected by maternal exposure to TPT. In conclusion, data provided by this study indicate that maternal treatment with TPT during pregnancy and lactation delayed prenatal growth but did not impair postnatal development and fertility in exposed offspring in adulthood.

Organotin compounds (OTC) are widely used as fungicides and acaricides in agriculture, as antifouling agents in paints for boats, as wood preservatives, as algicides, as molluscicides, and as disinfecting agents in industrial cooling waters. Moreover, some OTC have also been used as catalysts and plastic stabilizers in the industry (Fait et al., 1994). However, owing to OTC immunotoxicity and putative endocrine-disrupting properties, concern has grown about risks posed by these tin-based compounds to the environment and human health. In fact, several OTC were withdrawn from the market or their use was drastically restricted. Triorganotins, such as tributyltin (TBT), triphenyltin (TPT), and cyhexatin, are potent biocides and generally more toxic than mono-, di-, and tetra-substituted tin compounds (Kimbrough, 1976; Boyer, 1989).

Fentin or triphenyltin (TPT) has been widely used as a fungicide and miticide. In Brazil, the use of TPT-based plant protection products is currently authorized for a variety of crops such as cotton, garlic, peanut, rice, potato, cocoa, carrot, and common beans. Human exposure to OTC may occur due to occupational exposure or, in the case of the general population, through ingestion of OTC residues in food, particularly in fish and seafood. Mammalian toxicity attributed to TBT and TPT is well established, affecting the immune and reproductive systems (Boyer,

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1989; Fait et al., 1994; Benya, 1997; Ogata et al., 2001; Golub & Doherty, 2004). Since TBT and TPT were considered potential endocrine disruptors, there has been a renewed interest due to their adverse effects on mammalian reproduction (Golub & Doherty, 2004; Nakanishi, 2008). Grote et al. (2006) found that TPT impaired reproductive performance of the Japanese quail (*Coturnix coturnix japonica*). Recently exposure to TPT during pregnancy and lactation was shown to delay rat postnatal development in exposed offspring (Grote et al., 2009). Grote et al. (2009) demonstrated that TPT markedly reduced weight gain and delayed sexual maturation of male offspring, while the only effect noted in female offspring was a precocious vaginal opening. Thus far, studies on influence of TPT on reproductive toxicity have been conducted primarily in rats and rabbits. In the present study the reproductive toxicity of TPT was examined in the mouse. Previously, Sarpa et al. (2007) found that prenatal exposure to TPT enhanced embryo lethality and produced a dose-related increase in the occurrence of fetal skeleton anomalies in mouse fetuses at term. Subsequently, prenatal exposure to TPT was shown to impair the response of adult mice to malaria infection (Delgado et al., 2009). This study was undertaken to investigate further the effects of continuous maternal exposure to TPT during pregnancy and lactation on postnatal development as well as fertility parameters in mouse offspring surviving to adulthood.

MATERIALS AND METHODS

Animals

Male and virgin female Swiss Webster (specific-pathogen-free) mice from the Oswaldo Cruz Foundation Central Animal House breeding stock were used. Mice were housed individually in standard plastic cages with stainless-steel covers and with autoclaved wood shavings as bedding. Mice were kept under controlled temperature (23 ± 2°C), relative humidity (approximately 70%), and light/dark cycle (lights on from 8.00 to 20.00). A pellet diet (Nuvital, for laboratory rats and mice, Nuvilab Ltd, Curitiba, PR, Brazil) and filtered tap water were provided ad libitum. Experiments were conducted in accordance with internationally accepted ethical principles and Brazilian Animal Protection and Welfare laws. The study protocol (P0077-01) was approved by the Ethics Committee on the Use of Laboratory Animals of Oswaldo Cruz Foundation (CEUA-FIOCRUZ).

Mating Procedure

Mating was performed by placing 2 females into the cage of 1 male for 2 h at the end of the dark period (6:00–8:00 a.m.). The day on which copulation was confirmed by the presence of a vaginal plug was designated as d 0 of pregnancy.

Treatment

Fentin hydroxide (TPT, triphenyltin hydroxide; CAS number 76-87-9; ≥96.0% pure) was purchased from Sigma-Aldrich, Corp., St. Louis, MO. TPT dissolved in canola oil was administered by gavage [0 (control), 1.875, 3.75, or 7.5 mg/kg body weight/d] from pregnancy day 6 until weaning on postnatal day 21 (PND21). An untreated control group was evaluated as well. The number of pregnant females per dose group was as follows: untreated controls, 12; vehicle control, 18; 1.875 mg TPT, 17; 3.75 mg TPT, 18; and 7.5 mg TPT, 18. Dams were weighed on gestation days (GD) 0, 6–18, and during lactation until PND21.

Evaluation of Postnatal Growth and Somatic Maturation

From GD18 onward, dam cages were inspected twice a day (8 a.m. and 5 p.m.) for births, and the day on which they delivered their pups was designated as PND1. As soon as possible after birth the number of viable and dead newborns per litter was recorded, and all pups were sexed and weighed. Females not delivering until GD22 and those that cannibalized the whole litter were killed by CO_2 inhalation and autopsied. Pregnancy of females not delivering until GD22 was confirmed by the presence of implantation sites (determined by

the Salewski's method) in their uteri (Salewski, 1964). Maternal liver, spleen, thymus, ovaries, and uterine weights were recorded. Body weight gain of the offspring was recorded on PND 1, 5, 10, 15, 20, and 25. All pups were examined daily and the day on which developmental milestones appeared was recorded as follows:

Ear unfolding: first sign of detachment of the external pinnae of the ear from the side of the head.
Incisor eruption: first sign of eruption through the gums of both lower incisors.
Development of the fur: first detection of downy hair.
Eye opening: separation of upper and lower eyelids and complete opening of both eyes.
Vagina opening: first sign of detachment of the vagina.
Testes descent: first sign of testis descent, determined by scrotum palpation.

At weaning (PND21) all mothers were killed by CO_2 inhalation and autopsied.

Evaluation of Fertility in Adulthood

Weaned pups were housed (5 of the same gender per cage) in mouse standard cages until they were approximately 70 d old. Fertility tests were then carried out as follows. One male and 3 females from the same treatment group and different litters (to avoid brother–sister mating), chosen at random, were housed in the same cage for 15 d. After the mating period, females were transferred to individual cages. From GD18 onward cages were inspected twice a day for parturition. On PND1 the male and female live pups per litter were counted and mothers were killed by CO_2 inhalation. Mothers were autopsied and the number of implantation sites was determined by the method of Salewiski (1964). Females that had not been impregnated by males during the first cohabitation period were placed again into a male cage for a second mating test. The second mating test was similar to the first, except that untreated males of proven fertility were used. Males that did not impregnate at least 2 females during the first mating test had a second 15-d cohabitation period with 3 untreated females. One male mouse from each litter (chosen at random among those not used in fertility tests) was killed for collection of sperm from cauda epididymis. Collection of epididymal sperm and sperm counting was performed as described by Reddy et al. (2006).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test, or by the Kruskal–Wallis test followed by the Mann–Whitney *U*-test, whenever the data did not fit a normal distribution. Proportions were evaluated by the chi-square test or by Fisher's exact test. Statistical evaluation was performed using an SPSS program, and differences were considered as statistically significant at the level of $p < .05$.

RESULTS

The upper limit of the dose range tested in this study was set at 7.5 mg/kg bw/d because in previous investigations it was noted that at higher doses TPT induced maternal deaths as well as a marked prenatal and neonatal lethality in Swiss Webster mice (Sarpa et al., 2007; Delgado et al., 2008). Results from this mouse study showed that, except for a significant increase in liver relative weight at the highest dose, administration of TPT during pregnancy and lactation did not produce any discernible adverse effect on the dams. As shown in Table 1, TPT did not alter maternal body weight gain during pregnancy (GD18–GD0) and lactation (PND21–PND1). Moreover, behavioral changes and other signs of toxicity including mortality were not observed in mothers of TPT-treated groups.

Treatment with TPT from GD6 onward did not affect the duration of pregnancy (Table 1). All pups in 3 litters of the highest dose group (7.5 mg/kg; 18 litters in total) died on the day of birth (PND1) (Table 1). Since only remains were found in the cages, it was not possible to

TABLE 1. Effects of Fentin Administered Orally to Mice During Gestation and Lactation on Maternal Body Weight and Organ Weight, Duration of Pregnancy, Newborn Mortality, and Litter Size

Parameter	Untreated controls	Fentin (mg/kg body weight/d po)			
		0	1.875	3.75	7.5
Dams (*n*)					
GD 0	12	18	17	18	18
PND 1	12	18	17	18	18
PND 21	12	18	17	18	15
Entire litter deaths (%)[a]	0/12 (0)	0/18 (0)	0/17 (0)	0/18 (0)	3/18 (16.6) **
Gestation length[b]	19 (19–20)	19 (18–20)	19 (19–19)	19 (18–19)	19(18–19)
Body weight, g					
GD0	30.5 ± 2.7	31.2 ± 1.9	30.6 ± 3.2	30.0 ± 1.8	30.1 ± 2.4
GD18	62.7 ± 7.8	63.3 ± 5.6	65.5 ± 5.8	61.3 ± 4.5	61.3 ± 5.9
Weight gain, Δg					
GD 18-0	32.3 ± 6.3	32.1 ± 4.4	34.9 ± 3.4	31.3 ± 3.3	31.3 ± 4.7
PND 21-1	8.2 ± 3.2	6.1 ± 3.2	6.46 ± 4.3	6.1 ± 3.1	7.8 ± 2.8
Organ weight					
Liver (g)	3.1 ± 0.5	3.2 ± 0.3	3.3 ± 0.5	3.1 ± 0.3	3.4 ± 0.4*
(%)	6.4 ± 0.5	6.5 ± 0.6	6.7 ± 0.6	6.5 ± 0.5	7.0 ± 0.4**
Spleen (g)	0.22 ± 0.13	0.23 ± 0.07	0.23 ± 0.07	0.19 ± 0.06	0.19 ± 0.06
(%)	0.44 ± 0.24	0.47 ± 0.15	0.46 ± 0.12	0.40 ± 0.11	0.39 ± 0.10
Thymus (g)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
(%)	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.03	0.05 ± 0.03	0.04 ± 0.03
Litter size (*n*)					
PND1	10.1 ± 1.7	10.9 ± 2.0	11.6 ± 2.6	10.7 ± 1.6	10.8 ± 2.2
PND21	9.8 ± 1.6	10.9 ± 2.0	11.1 ± 2.4	10.0 ± 1.9	9.5 ± 2.6

Note. *n*, Number; GD, gestation day; PND, postnatal day. Data shown as means ± SD were analyzed by ANOVA followed by Bonferroni's test. Medians (range) were compared by the Kruskal–Wallis test. Proportions (%) were compared by the chi-square test. Significant differences ($p < .05$) are indicated by asterisks: (*) from control, (**) from controls and lower dose groups.

[a]On PND1, all pups from three litters were cannibalized by their mothers.

[b]Duration of pregnancy = days (median; range) elapsed between GD0 and the day on which pups were spontaneously delivered.

ascertain whether (1) the three mothers cannibalized pups that had died spontaneously due to treatment-related stillbirths or newborn deaths, or (2) the dams killed and subsequently ate their offspring. Although there was a higher incidence of whole-litter deaths on PND1 at 7.5 mg/kg, maternal exposure to TPT during pregnancy at this dose did not reduce the size of the surviving litters, thereby suggesting that TPT did not increase pre- and or neonatal lethality within litters (Table 1). Exposure to TPT, however, produced a significant 5–10% dose-related decrease in pup body weight at birth (PND1) (Table 2). It is noteworthy that significant reductions of pup body weight at birth were found at doses of TPT that were not overtly toxic to mothers. Results from this study therefore suggested that TPT was embryotoxic, as evidenced by reduced newborn body weight on PND1 at doses that were not maternally toxic. As shown in Table 1, litter size of TPT-exposed groups on the day of weaning (PND21) did not differ from those of untreated and vehicle-treated controls. This finding indicated that maternal exposure to TPT over pregnancy and the entire lactation period did not enhance pup mortality from PND1 until PND21. Furthermore, maternal exposure to TPT exerted no apparent effect on the postnatal growth and maturation of the offspring.

Although exhibiting a lower body weight at birth (PND1), offspring from dams treated with TPT showed no body weight loss compared to controls on PND5 and thereafter (Table 2). There was also no discernible effect of maternal treatment on the day of appearance of landmarks of physical maturation such as ear detachment, fur development, incisor eruption,

TABLE 2. Postnatal Body Weight Development (g) of Offspring From Mice Treated Orally With Fentin During Gestation and Lactation

Parameter	Untreated controls	Fentin (mg/kg body weight/d po)			
		0	1.875	3.75	7.5
Litters (*n*)	12	18	17	18	15
PND					
1	1.79 ± 0.15	1.76 ± 0.13	1.67 ± 0.11*	1.66 ± 0.11*	1.57 ± 0.12**
5	2.96 ± 0.40	2.94 ± 0.28	2.95 ± 0.39	2.98 ± 0.34	2.87 ± 0.28
10	5.30 ± 0.94	5.01 ± 0.69	5.03 ± 0.67	5.19 ± 0.64	4.96 ± 0.71
15	6.98 ± 1.43	6.61 ± 0.92	6.53 ± 1.00	6.63 ± 1.04	6.61 ± 1.15
20	9.42 ± 2.51	8.48 ± 1.51	8.43 ± 1.65	8.24 ± 1.85	8.56 ± 1.97
25	15.22 ± 3.86	14.11 ± 2.09	14.00 ± 2.73	14.19 ± 3.21	14.50 ± 3.53
80	48.7 ± 4.00	48.7 ± 3.4	49.0 ± 5.76	48.7 ± 5.2	47.7 ± 5.2

Note. n, number. Data (means ± SD) were analyzed by ANOVA followed by Bonferroni's post hoc test. Significant differences ($p < .05$) are indicated by asterisks: (*) from control, (**) from controls and lower dose groups. Pups were weaned on PND 21. The litter was the unit of analysis.

eye opening, and testes descent or vaginal opening (data not shown). Fertility tests with sexually mature mice (>70 d old) did not reveal any difference between offspring from mothers exposed to TPT during gestation and lactation and those from controls (data not shown). No marked differences between controls and TPT-treated groups regarding epididymal sperm counts were found (data not shown). Weight of testes and epididyms, measured at the end of the study (PND80–90), did not differ significantly between controls and TPT-exposed groups either (data not shown).

DISCUSSION

Data provided in this study showed that exposure to TPT at 7.5 mg/kg during pregnancy (from GD6 onward) increased the number of whole litter deaths, reduced pup body weight at birth, and increased maternal liver weight. No other effects were noted at this dose level and no adverse effects on mothers and their offspring were noted at doses lower than 7.5 mg/kg /d po. Since mean litter size at birth (PND1) remained unchanged, it is apparent that at doses tested, TPT did not increase within-litter mortality, and thus this chemical did not produce embryofetal deaths, stillbirths, or newborn deaths (PND1) in the surviving litters. Only remains of partially cannibalized pups were found in the cages of dams that lost their litters. Under those circumstances, it was not possible to ascertain whether mothers cannibalized stillborn and pups that died soon after birth, or pups were killed by their mothers (infanticide behavior). In a previous study with the same strain of mice, TPT at doses equal to and greater than 7.5 mg/kg bw/d po on GD6–18 enhanced the occurrence of fetal anomalies such as misshapen thymus, misshapen palatine rugae, and cleft palate (Sarpa et al., 2007). Since female mice frequently kill their malformed, ill, or less viable pups, whole-litter deaths noted at the highest dose of TPT might have been due to maternal infanticide behavior triggered by the presence of malformed pups in the litters.

The lower pup body weight at birth showed that TPT, at doses >1.875 mg/kg /d, retarded prenatal growth, an indication of embryotoxicity. It is noteworthy that TPT reduced pup birth weight at doses that were not maternally toxic. These results and those from a previous study in Swiss Webster mice (Sarpa et al., 2007) suggested that TPT is a selective developmental toxicant.

Although there was a decrease in mouse pup birth weight, maternal treatment with TPT during pregnancy and lactation did not impair postnatal weight gain and acquisition of landmarks of somatic development such as ear unfolding, fur development, incisors eruption, eye opening, testes descent, and vagina opening. Results therefore indicated that continuous maternal exposure to TPT during pregnancy

and lactation adversely affected prenatal but not postnatal development of offspring. In addition, data from this study demonstrated that exposure to TPT during gestation and lactation did not impair the performance of sexually mature mice (>70 d old) in fertility tests (data not shown). It should be borne in mind, however, that only marked reductions of the number of fertile sperm would be detected as a decrease in the reproductive capacity in offspring. It is known that the number of sperm in the mouse ejaculate usually exceeds by far the number needed for normal fertility and litter size (Amann, 1986). Reddy et al. (2006) reported that a low dose of TPT given intraperitoneally (3 × 25 μg/kg /d ip) impaired spermatogenesis and markedly reduced sperm quantity and quality, decreased weights of testes and epididymis, and lowered testosterone blood levels in adult (aged >50 d old) Swiss mice. Since these changes were found 20 d after the last ip injection, results by Reddy et al. (2006) indicated that TPT produced a persistent impairment of spermatogenesis in adult mice. Our results with TPT are apparently at variance with those of Reddy et al. (2006). It should be noted, however, that routes (direct exposure by ip injections vs. transplacental and milk transfer after maternal oral exposure), time (adulthood vs. embryofetal and postnatal development), and lengths of exposure (3 doses in 5 d vs. gestation and suckling periods) were quite different between the two studies. As far as we are aware, there is no other study providing evidence that OTC impair male reproductive function in exposed mice. Some studies in rats found that oral exposure to TBT during very early pregnancy blocked implantation, thereby reducing female fertility (Ema et al., 1995; Harazono et al., 1996). A similar effect of TPT was described by Winek et al. (1978) in rats exposed orally to a dose as high as 20 mg/kg/d on GD 1–7. Since in this study treatment of female mice began on GD6, the presence of an inhibitory effect of TPT on implantation was not expected. Futhermore, our results with offspring of mice exposed to TPT (1.875–7.5 mg/kg) are at variance with those recently reported by Grote et al. (2009) for offspring of rats treated with TPT (2–6 mg/kg/d) during pregnancy and lactation. Grote et al. (2009) found that, while significantly reducing body weight gain and sexual organ weights and delaying preputial separation in the male offspring, treatment with TPT produced only a precocious vaginal opening in female offspring. The underlying causes of this gender difference in the response of rat offspring to TPT were not clarified by Grote et al. (2009). Contrasting with data on rats, our results suggest that postnatal development of male and female mouse offspring remains unaffected by maternal exposure to TPT during gestation and lactation.

A kinetic study in rats demonstrated that TBT (tributyltin) chloride crosses the placenta and accumulates in the embryo. During lactation, however, only minimal amounts of this OTC were transferred to pups through mothers' milk (Cooke et al., 2008). Another rat study also found that while there was a substantial transplacental transfer of Sn from DBT (dibutyltin) to offspring, transfer of tin (Sn) to pups via maternal milk was almost negligible (Moser et al., 2009). A number of other kinetic studies in mammals exposed to OTC have consistently shown that Sn is transplacentally transferred to embryos and fetuses. Although no similar kinetic data are available for mice, findings from the present study showing that TPT, at doses that were not toxic to dams, reduced pup body weight at birth supporti the view that TPT and/or its metabolites are also transplacentally transferred to the mouse offspring. The lack of effects of maternally administered TPT on postnatal growth and maturation of offspring is consistent with the interpretation that mouse, similar to rat, pups received only minimal amounts, if any, of these compounds through dam milk.

In conclusion, results from this study showed that mouse exposure during pregnancy to TPT reduced pup body weight at birth at doses greater than 1.875 mg/kg/d and increased neonatal mortality in some 3 out of 18 dams (whole-litter deaths) at 7.5 mg/kg/d. Further maternal exposure to TPT during lactation, however, produced no (1) pup body weight loss from PND 5 onward, (2) retardation

of physical maturation, or (3) impairment of fertility in adulthood. Based on results presented in this study, the no-observed-adverse-effect level (NOAEL) for TPT-induced maternal toxicity was found to be 3.75 mg/kg/d (liver enlargement), while the NOAEL for developmental toxicity was higher than 1.875 mg/kg/d (lower birth weight). It is noteworthy that TPT was toxic to the embryo at doses that produced no evidence of maternal toxicity.

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