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Developmental toxicity of triphenyltin hydroxide in mice

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Abstract

Triphenyltin-hydroxide (TPTH) is used as agricultural fungicide in Brazil and elsewhere. This study was undertaken to evaluate the developmental toxicity of TPTH in mice. Swiss Webster mice were treated by gavage with TPTH (0, 3.75, 7.5, 15 and 30 mg/kg bw/day) on gestation days (GD) 6–17. Caesarean sections were performed on GD 18, and implantations, resorptions and live and dead fetuses were counted. Half of each litter was fixed and examined for visceral anomalies while the remaining fetuses were cleared and stained with Alizarin Red S for skeleton evaluation. A reduced pregnancy weight gain (after subtraction of uterine weights), smaller thymus, spleen and liver, and deaths indicated that doses $\geqslant$7.5 mg/kg body wt/day were toxic to mothers. At the two highest doses, TPTH enhanced embryolethality and reduced fetal body weight. The incidence of cleft palate (not seen in controls) was augmented (36.8%) at the highest dose of TPTH, while palatine bone defects were increased at the lowest dose (3.75 mg/kg bw/day). Soft-tissue anomalies, such as misshapened thymus, and malpositioned testes and uteri, were more frequent at doses of TPTH $\geqslant$7.5 mg/kg bw/day. TPTH also caused a dose-related increase of fetal skeleton variations (e.g. poorly ossified skull bones) and malformations (misshapened Axis and skull bones). In conclusion, TPTH was toxic to the embryos (NOAEL <3.75 mg/kg bw/day) at doses that were not overtly toxic to their mothers.

Keywords: Fentin hydroxide; TPTH; Organotin compounds; Pre-natal toxicity; Teratogenicity; Embryotoxicity

1. Introduction

Triorganotin compounds (OTCs) have been used as fungicides, bactericides, antihelminthics, miticides, herbicides, molluscicides, insecticides, nematocides, rodent repellents, and antifouling agents in boat paint (Fent, 1996; WHO (World Health Organization) and IPCS (International Program in Chemical Safety), 1980; WHO (World Health Organization) and IPCS (International Program in Chemical Safety), 1999). Triphenyltin hydroxide (TPTH, CAS 76-87-9), also known as fentin hydroxide, is an OTC used as an agricultural non-systemic fungicide. In Brazil, TPTH is currently allowed for cotton, cocoa, garlic, onion, potato, rice, carrot, peanut, and bean crops (ANVISA (Agência Nacional de Vigilância Sanitária), 2006).

OTCs are markedly toxic to oysters and other non-target mollusks and have been considered as environmental endocrine-active chemicals. For instance, tributyltin (TBT) and triphenyltin (TPT) are inducers of imposex, or imposition of male sex characteristics on female snails (Fent, 1996; Oberdorster and McCClellan-Green, 2002). The mechanism by which these OTCs cause imposex remains to be elucidated but OTC-induced inhibition of an aromatase, a cytochrome-P450 that converts testosterone into estradiol, seems to be involved (Oberdorster and McCClellan-Green, 2002).

Toxicity of OTCs to vertebrate species has been cause for concern as well. Studies in rodents have shown that TBT and TPT are absorbed in the gastro-intestinal tract, and that levels of TPT and its metabolites in the liver and kidneys are

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higher than levels in the blood and other tissues (Ohhira and Matsui, 1996; Appel, 2004). Although OTCs apparently do not possess genotoxic or carcinogenic properties, they are markedly toxic to mammalian nervous and immune systems. OTCs have been shown to impair T-cell-dependent humoral (IgM and IgE) and cellular immune responses and to produce lymphopenia and atrophy of lymphoid organs such as thymus and spleen (WHO (World Health Organization) and IPCS (International Program in Chemical Safety), 1999). Neurotoxicological effects, such as neuronal necrosis and maze learning test deficits, have also been reported in rodents exposed to triorganotins (WHO (World Health Organization) and IPCS (International Program in Chemical Safety), 1999).

The effects of TBT and TPT on mammalian reproduction, however, are far from being entirely understood. Developmental toxicity of triorganotin compounds has been evaluated in a few rodent and rabbit studies. TPT and TBT chloride were reported to prevent blastocyst implantation in rats (Ema et al., 1997, 1999). While TPTH (Winek et al., 1978) and triphenyltin acetate (TPTA) (Giavini et al., 1980; Noda et al., 1991a) were described not to be teratogenic in rats, bis(tri-*n*-butyltin)oxide (TBTO) was found to be embryotoxic and to cause cleft palate in mice (Baroncelli et al., 1990; Davis et al., 1987). An increased incidence of cleft palate was also noted in rat fetuses exposed to either TBTO (Crofton et al., 1989) or tributyltin acetate (TBTA) (Noda et al., 1991b) during gestation. Lower fetal body weight and delayed ossification, but no evidence of teratogenicity, were noted in the rat offspring prenatally exposed to tributyltin chloride (TBTCl, 20 mg/kg) (Adeeko et al., 2003). Recently, Tsukamoto et al. (2004) reported that prenatal exposure to TBTCl (1 mg/kg bw sc) inhibited calcification of supraoccipital bone in mice. The authors also showed that, in culture of rat calvarial osteoblast cells, TBTCl reduced the activity of alkaline phosphatase (ALPase), the rate of calcium deposition, and the levels of expression of m-RNA for ALPase and osteocalcin (markers of osteoblastic differentiation) (Tsukamoto et al., 2004). As far as we are aware, there is no study on the effects of TPT on the ossification of the mouse skeleton.

A key issue regarding the developmental toxicity of TPT that has also remained unanswered by rat and rabbit studies performed so far is whether embryotoxicity occurs secondary to maternal effects or occurs also in the absence of maternal toxicity.

This study was undertaken to evaluate the developmental toxicity of TPTH in the mouse.

2. Materials and methods

2.1. Animals

Male and nulliparous female Swiss Webster mice (50–60 days old, weighing 30–35 g) from the Oswaldo Cruz Foundation breeding stock were used. The mice were housed individually in standard plastic cages with stainless-steel covers and wood shavings as bedding and kept under controlled temperature (23 ± 2 °C), relative humidity (approximately 70%) and 12:12 h photoperiod with lights on at 0800 h. A standard commercial diet for laboratory rats and mice (CR1 Nuvital®, Nuvilab Ltd., Curitiba, PR, Brazil) and tap water were provided *ad libitum*. The research protocol was approved by the "Ethics Committee on the Use of Laboratory Animals of Oswaldo Cruz Foundation" (CEUA-FIOCRUZ).

2.2. Mating procedure

For mating, two females were placed into the cage of one male mouse for 2 h at the end of the dark period (6:00–8:00 h). The day on which copulation was confirmed by the presence of a vaginal plug was designated as day '0' of pregnancy.

2.3. Treatment

Triphenyltin hydroxide (97.3% pure; AGREVO Ltd., Brazil) (CAS Registry no 76-87-9) was suspended in corn oil (Mazola®) and administered (0, 3.75; 7.5; 15.0 and 30.0 mg/kg bw/day) by gavage to mice once a day on days 6–17 of pregnancy. The control group received an equivalent volume of corn oil (10 mL/kg bw/day) alone. All mice were weighed on day 0 and on a daily basis on GD 6–18.

2.4. Caesarean section

On day 18 of pregnancy the female mice were killed by CO_2 inhalation. The gravid uteri were weighed with their contents. The number of dead and living fetuses and resorptions were recorded. The number of implantation sites was determined by the Salewski's method (Salewski, 1964). Fetuses were weighed, examined for externally visible abnormalities and numbered with a marker pen. Half of the fetuses from each litter, selected at random, was fixed in Bouin's solution and examined for visceral anomalies by using a microsectioning technique adapted from Sterz (1977). The remaining fetuses were fixed in a 5% formalin solution, eviscerated, placed in a solution of ethyl ether: 99% ethanol (1:4) for 7 days, and rinsed in running water for one day. Skeletons were then stained in Alizarin Red S (0.3% alizarin in KOH 10% w/v) for 7–8 days, rinsed in running water for one day, cleared in a solution of benzyl alcohol: 99% ethanol: 80% glycerol (1:2:2) for one day, and stored in 100% glycerol (adapted from Dawson, 1926).

2.5. Statistical analysis

Data were analyzed by the one-way analysis of variance (ANOVA) or by the Kruskal–Wallis test whenever they did not fit a normal distribution. Differences between groups were further tested by the Duncan's test or by the Mann–Whitney *U* test, respectively. Proportions were evaluated by the chi-square test. The Fisher exact test was used as an alternative to the chi-square test if any expected frequency (in 2×2 contingency tables) was smaller than 5. Statistical calculations were performed using a MINITAB program (MTB, University of Pennsylvania, 1984), and differences were considered as significant when $P < 0.05$.

3. Results

3.1. Maternal toxicity

The effects of TPTH on pregnancy weight gain and on the weight of maternal organs are shown in Table 1. At the two highest doses tested (15 and 30 mg/kg bw/day), TPTH markedly reduced maternal weight gain (GDs 6–11, 6–15 and 15–18). The decrease of overall weight gain (GDs 0–18) resulted—to a great extent—from a harmful effect on the mother because it was even more evident when

gravid uterus weight at term was subtracted from the weight gain during whole pregnancy (Table 1). Maternal toxicity of the two highest doses of TPTH was additionally demonstrated by a pronounced decrease of liver, spleen and thymus weights (Table 1). The deaths of two dams treated with the highest dose (on GD 17 and 18) and one dam treated with the second highest dose (on GD 18) also showed that TPTH was maternally toxic at these dose levels. All maternal deaths noted at the two highest doses were preceded by clinical symptoms such as apathy, marked loss of hair (alopecia), decreased food intake and drastic reduction of body weight. The effect of an intermediate dose of TPTH (7.5 mg/kg bw/day) on pregnancy weigh gain was less evident but, in this case, a slight reduction of mother's body weight gain was detected when the uterine weight was subtracted from dam's overall weight gain. Weights of maternal liver and spleen were also diminished by treatment with 7.5 mg of TPTH per kg bw per day on GD 6–17 (Table 1) thereby confirming that this intermediate dose was toxic to the dams. Neither pregnancy weight gain nor maternal organ weights, however, were altered by TPTH at the lowest dose tested (3.75 mg/kg bw/day). One dam treated with the lowest dose (3.75 mg/kg bw/day) was found dead on GD 14. This death on the 8th day of treatment was atypical as compared to those caused by higher doses of TPTH because it was not preceded by a reduction of body weight gain and other signs of toxicity. No alteration of maternal organs other than thymus, spleen and liver was noted at the cesarean section. The foregoing results clearly indicated that TPTH produced maternal toxicity at doses equal to and higher than 7.5 mg/kg bw/day.

3.2. Embryotoxicity

Neither the ratio of pregnant (females with uterine implantations) per treated (sperm plug positive) females (Table 1), nor the number of implantation sites detected by the method of Salewski was altered by TPTH (Table 2). Since in the mouse implantation takes place on GD 4.5–6 (Leone, 1977), these findings indicated that TPTH administered from GD 6 on did not induce peri-implantation embryo losses. Nonetheless, an increased occurrence of resorptions as well as a smaller number of live fetuses demonstrated that the two highest doses of TPTH (15 and 30 mg/kg bw/day) were embryolethal (Table 2). TPTH produced increases in the frequency of early resorptions (Table 2), thereby indicating that most embryos died soon after implantation, during the first days of treatment. Fetal sex ratios for treated groups did not differ from that of the control group, a finding indicative that the lethal effect of TPTH affected to the same extent male and female embryos (Table 2). Near term fetal body weight was reduced at the two highest doses, a result suggestive that, at doses higher than 7.5 mg/kg bw/day, TPTH retarded the growth of the surviving embryos (Table 2).

Except for an increased occurrence of edema at the highest dose group, no difference was found between control and TPTH-exposed fetuses regarding the frequency of externally-visible abnormalities (Table 3). Nevertheless, visceral examinations revealed a markedly higher frequency of cleft palate in TPTH-exposed fetuses, particularly in those of the highest dose group (Table 3). Noteworthy, in the groups exposed to doses of TPTH equal to or higher than 7.5 mg/kg bw/day,

Table 1
Maternal weight gain of mice treated orally with triphenyltin hydroxide (TPTH, 0, 3.75, 7.5, 15.0 and 30.0 mg/kg bw/day) on days 6–17 of pregnancy

Treatment	TPTH (mg/kg bw/day)				
	0	3.75	7.5	15	30
Treated females (*N*)	22	23	22	23	21
Pregnant females (*N*)[+]	20	21	19	21	17
Pregnant/treated females (%)	90.9	91.3	86.4	91.3	81.0
Maternal body weight (g)					
Day 0	31.9 ± 2.0	32.1 ± 3.3	31.0 ± 2.4	31.5 ± 1.8	32.8 ± 2.5
Day 18	62.1 ± 8.4	62.3 ± 6.2	58.2 ± 9.7	48.2 ± 13.0[a,b,c]	41.5 ± 10.9[a,b,c]
Gravid uterus weight (g)	20.4 ± 6.4	20.7 ± 3.4	19.8 ± 6.4	14.20 ± 9.2[a,b,c]	8.3 ± 9.5[a,b,c,d]
Maternal weight gains (g)					
Day 6–0	4.3 ± 2.0	4.6 ± 1.5	4.2 ± 2.1	4.4 ± 1.2	3.9 ± 1.3
Day 11–6	5.1 ± 1.6	4.9 ± 1.2	4.9 ± 1.1	1.3 ± 3.0[a,b,c]	-0.8 ± 3.3[a,b,c]
Day 15–6	15.4 ± 3.9	14.7 ± 3.4	14.6 ± 4.1	7.0 ± 7.6[a,b,c]	3.5 ± 6.6 [a,b,c]
Day 18–15	10.5 ± 3.6	10.9 ± 3.4	8.5 ± 4.8	4.9 ± 5.8[a,b,c]	1.1 ± 4.1[a,b,c,d]
Day 18–0	30.2 ± 8.5	30.2 ± 4.2	27.3 ± 9.2	16.7 ± 12.6[a,b,c]	8.6 ± 10.7[a,b,c]
Day 18–0 (minus uterus weight)	9.8 ± 3.1	9.4 ± 2.3	7.4 ± 4.1[a]	2.5 ± 5.0[a,b,c]	0.2 ± 2.6[a,b,c]
Weight of maternal organs (g)					
Spleen	0.17 ± 0.05	0.15 ± 0.07	0.13 ± 0.03[a,b]	0.09 ± 0.03[a,b]	0.08 ± 0.03[a,b,c]
Thymus	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.01 ± 0.01[a]	0.01 ± 0.01
Liver	3.24 ± 0.48	3.20 ± 0.34	2.94 ± 0.62[a,b]	2.64 ± 0.56[a,b,c]	2.53 ± 0.41[a,b,c]

[+] Pregnant females, pregnancy verified by the presence of uterine implantation sites. Data shown as means ± SD were analysed by ANOVA followed by the Duncan's test. Proportions (%) were compared by the chi-square test. Differences ($P < 0.05$) between dose levels are indicated by superscripts as follows: a ≠ 0 mg/kg, b ≠ 3.75 mg/kg, c ≠ 7.5 mg/kg, d ≠ 15 mg/kg.

Table 2
Cesarean section data of mice treated orally with triphenyltin hydroxide (TPTH, 0, 3.75, 7.5, 15.0 and 30.0 mg/kg bw/day) on days 6–17 of pregnancy

Treatment	TPTH (mg/kg bw/day)				
	0	3.75	7.5	15.0	30.0
Implantation sites					
Total (*N*)	264	252	253	259	187
N per litter (mean ± SD)	13.2 ± 2.7	12.6 ± 2.3	13.3 ± 2.3	13.0 ± 1.8	12.5 ± 2.7
Resorptions					
Total (*N*)	30	16	32	77	109
Per implantation sites (%)	11.4	6.4[a]	12.6[b]	29.7[a,b,c]	58.3[a,b,c,d]
N per litter (mean ± SD)	1.5 ± 1.8	0.8 ± 1.1	1.7 ± 2.4	3.8 ± 5.2[b]	7.3 ± 6.2[a,b,c,d]
Early (*N*)	9	1	11	64	101
Median (range)	0 (0–7)	0 (0–1)	0 (0–9)	0 (0–14)	7 (0–15)[a,b,c]
Intermediate (*N*)	17	7	18	7	5
Median (range)	0 (0–5)	0 (0–2)	0 (0–5)	0 (0–2)	0 (0–2)
Late (*N*)	4	8	3	6	3
Median (range)	0 (0–1)	0 (0–4)	0 (0–2)	0 (0–2)	0 (0–1)
Resorption of the whole litter (*N*)	1	0	1	5	8
Dead fetuses	2	2	4	4	0
Live fetuses					
Total (*N*)	234	238	217	179	80
Per implantation sites (%)	88.6	94.4[a]	85.8[b]	69.1[a,b,c]	42.8[a,b,c,d]
N per litter (mean ± SD)	11.7 ± 3.9	11.9 ± 2.1	11.4 ± 4.1	9.0 ± 5.6	5.3 ± 6.2[a,b,c,d]
Fetal body weight (g)					
Per litter (mean ± SD)	1.31 ± 0.07	1.30 ± 0.08	1.30 ± 0.16	1.12 ± 0.21[a,b,c]	1.08 ± 0.12[a,b,c]
Sex ratio (F/M)	115/118	127/111	116/101	87/91	43/37

N, number. Proportions (%) were compared by the chi-square. Data shown as median (range) were evaluated by the Kruskal–Wallis test and Mann–Whitney *U* test, and those shown as means ± SD by the one-way ANOVA followed by the Duncan test. Differences ($P < 0.05$) between dose levels are indicated by superscripts as follows: a ≠ 0, b ≠ 3.75 mg/kg, c ≠ 7.5 mg/kg, d ≠ 15 mg/kg. Resorptions were classified as follows: early, implantation sites detected where there were neither fetuses nor visible fetal/placental remnants; intermediate, fetal/placental remnants were visible but discrimination between fetal and placental residues was not possible; late, discrimination between fetal and placental residues was still possible.

some fetuses with apparently fused palate shelves exhibited irregularly shaped (misshapened) palatine rugae (Table 3).

Increased occurrences of misshapened thymus, malpositioned testes and malpositioned uterus were also noted in fetuses exposed to doses of TPTH equal to or higher than 7.5 mg/kg bw/day (Table 3). Although misshapened palatine rugae, malpositioned heart, malpositioned kidney, smaller eye and misshapened liver were also more frequent among TPTH-treated fetuses, the increased occurrence of these anomalies was not dose dependent (Table 3).

The occurrence of skeletal abnormalities in fetuses exposed to TPTH on GDs 6–17 is shown in Table 4. Fetal skeleton examination revealed that TPTH caused a marked and dose dependent increase in the incidence of poorly ossified skull bones (e.g. *os basioccipitale*, *os basisphenoid*, *os frontale*, *os hamulus*, *os presphenoid*, *os lacrimale* and *os parietale*). TPTH produced a dose-related increase in the occurrence of poorly ossified sacral and caudal vertebrae as well (Table 4). Poor ossification of some forelimb bones (e.g. clavicle, scapula, ulna, humerus, radius) was also noted among TPTH exposed fetuses but, in all these cases, the increased occurrence was not related to the dose of the compound (Table 4). It should be pointed out that poorly ossified bones have generally been regarded as variations when anomalies are classified using the two categories scheme (variations and malformations) proposed by Chahoud et al. (Chahoud et al., 1999; Solecki et al., 2001). TPTH, at the highest dose tested (30 mg/kg bw/day), augmented the incidence of rudimentary lumbar extra ribs, an anomaly that has also been classified by most authors as a variation (Solecki et al., 2001).

An increased incidence of skull bone malformations was noted in TPTH-exposed fetuses. Frequencies of misshapened *os basioccipitale* and misshapened *os basisphenoid* were increased by TPTH but, in both cases, the anomaly was also recorded among control (vehicle-treated) fetuses (Table 5). It is of note that TPTH, at the two highest doses (15 and 30 mg/kg bw/day), increased the occurrence of some skull bone anomalies, such as misshapened *os interparietale*, bipartite *os supraoccipitale* (Fig. 1), misshapened *os praesphenoid* and misshapened *os pterygoid,* that were not observed in control fetuses (Table 5). The most striking effect of TPTH was a marked and dose dependent elevation of the incidence of skull bone anomalies related to a deficient fusion of palate shelves (cleft palate) such as a wider distance between palatine bones (Table 4, Fig. 1). Nonetheless, while a statistically significant increase of cleft palate was found only at the highest dose (Table 3), a wider distance between palatine bones—an anomaly not seen in control fetuses—was detected at the lowest dose of TPTH tested (3.75 mg/kg bw/day) (Table 4). Last, higher frequencies of malformations of the first (ossified cartilage and bent Atlas) and the second (misshapened Axis) cervical vertebrae, which were not observed in control fetuses, were recorded at the two highest doses of TPTH (Table 5).

Table 3
Occurrence of externally visible and visceral abnormalities in the offspring of mice treated orally with triphenyltin hydroxide (TPTH, 0, 3.75, 7.5, 15.0 and 30.0 mg/kg bw/day) on days 6–17 of pregnancy

Treatment	TPTH (mg/kg bw/day)				
	0	3.75	7.5	15.0	30.0
Externally-visible abnormalities					
Fetuses examined (*N*):	234	238	217	179	80
Litters examined (*N*):	19	20	18	15	7
Hematoma					
Fetuses (%)	0	0	0	0	1.2
Litters (%)	0	0	0	0	14.3
Edema					
Fetuses (%)	0	0	0	0	6.2
Litters (%)	0	0	0	0	28.6
Agnathia					
Fetuses (%)	0	0	0.5	0	0
Litters (%)	0	0	5.6	0	0
Exencephaly					
Fetuses (%)	0.4	0.4	0	0	1.2
Litters (%)	5.3	5.0	0	0	14.3
Exophthalmia					
Fetuses (%)	0.4	0	0	0	1.2
Litters (%)	5.3	0	0	0	14.3
Tail (rudimentary)					
Fetuses (%)	0	0	0	0.6	0
Litters (%)	0	0	0	6.7	0
(Kinky)					
Fetuses (%)	0.4	1.7	0	0	0
Litters (%)	5.3	15.0	0	0	0
Visceral anomalies					
Fetuses examined (*N*)	113	115	103	85	38
Litters examined (*N*)	19	20	18	15	7
Cleft palate					
Fetuses (%)	0	2.6	1.0	3.5[a]	36.8[a]
Litters (%)	0	15.0	11.1	13.3	71.4[a]
Palatine rugae (misshapened)					
Fetuses (%)	0	0	5.8[a]	3.5[a]	7.9[a]
Litters (%)	0	0	27.7[a]	20.0[a]	42.8[a]
Thymus (misshapened)					
Fetuses (%)	0	1.7	17.5[a]	29.4[a]	50.0[a]
Litters (%)	0	10.0	55.5[a]	80.0[a]	85.7[a]
Heart (malpositioned)					
Fetuses (%)	0	0	0.9	1.2	2.6
Litters (%)	0	0	5.5	6.6	14.3
(Misshapened + malpositioned)					
Fetuses (%)	0	0	0	0	2.6
Litters (%)	0	0	0	0	2.6
Kidneys (malpositioned)					
Fetuses (%)	0	1.7	0	2.3	7.9
Litters (%)	0	10.0	0	13.3	42.8
Ureter (malpositioned)					
Fetuses (%)	0	0	0.9	0	0
Litters (%)	0	0	5.5	0	0
Eye (smaller)					
Fetuses (%)	0	0	0.9	0	2.6
Litters (%)	0	0	5.5	0	14.3
Testes (malpositioned)					
Fetuses (%)	0	0	6.7[a]	15.3[a]	21.0[a]
Litters (%)	0	0	22.2	53.3[a]	57.1[a]
Uterus (malpositioned)					
Fetuses (%)	0	0.9	0	7.0[a]	23.7[a]
Litters (%)	0	5.0	0	33.3[a]	71.4[a]
Liver (misshapened)					
Fetuses (%)	0	1.7	0	0	2.6
Litters (%)	0	10.0	0	0	14.3
(Extra lobule)					
Fetuses (%)	0.9	3.5	0	1.2	0
Litters (%)	5.3	10.0	0	6.6	0

Proportions were analysed by the chi-square test. Differences ($P < 0.05$) are indicated by superscripts as follows: a ≠ 0, b ≠ 3.75 mg/kg, c ≠ 7.5 mg/kg, d ≠ 15 mg/kg. Heart anomaly categories "malpositioned" and "misshapened + malpositioned" are mutually exclusive.

4. Discussion

Results from this study showed that TPTH was toxic to pregnant mice at oral doses equal to or higher than 7.5 mg/kg bw/day. The embryotoxic effects of TPTH, however, were observed at all tested doses, that is, at 3.75 mg/kg bw/day and higher doses.

Although TPTH proved to be embryolethal over the dose range tested, it did not induce very early implantation or peri-implantation losses. Two previous studies found that early gestation exposure to OTCs prevented implantation in the rat. TPTCl (in olive oil, 4.7 mg/kg/day) given by gavage either on GD 0–3 or on GD 4–6 (Ema et al., 1997), and TBTCl (in olive oil, 16.3 mg/kg/day) given by gavage on GD 0–7 (Harazono et al., 1998), were reported to cause implantation failure in rats. A further study by Ema et al. (1999) suggested that OTC-induced failure of implantation in rats was due to lower progesterone levels and suppression of uterine decidualization.

A higher frequency of resorptions and a lower ratio of live fetuses per implantations both indicated that doses of TPTH equal to or higher than 15 mg/kg bw/day caused post-implantation embryo deaths. A lower fetal body weight at term (GD 18) suggested that doses of TPTH equal to or higher than 15 mg/kg bw/day delayed prenatal growth as well.

Embryotoxicity of TPTH was also revealed by an increased occurrence of a variety of soft tissue and skeletal abnormalities in the exposed fetuses. In the group exposed to TPTH, we found a higher incidence of fetuses with malpositioned viscera such as malpositioned testes, kidneys, uterine horns and, to a lesser extent, malpositioned heart. These findings are consistent with a higher frequency of malpositioned testes found by Ema et al. (1991) in rats exposed to a disubstituted organotin compound (di-*n*-butyl-tin chloride, 5 mg/kg bw/day) on GDs 7–15. Misshapened thymus, an anomaly not seen in control fetuses, increased in a dose-related manner among TPTH-exposed fetuses with a NOEL around 3.75 mg/kg bw thereby indicating that this lymphoid organ is one of the most sensitive targets for developmental toxicity of TPTH, a known immunotoxic agent (WHO (World Health Organization) and IPCS (International Program in Chemical Safety), 1999; Benya, 1997; Boyer, 1989).

Prenatal exposure to TPTH also caused a dose dependent increase in the frequency of poorly ossified bones in

Table 4
Occurrence of poorly ossified and unossified bones and wider distance between palatine bones in the offspring of mice treated orally with triphenyltin hydroxide (TPTH, 0, 3.75, 7.5, 15.0 and 30.0 mg/kg bw/day) on days 6–17 of pregnancy

Treatment	TPTH (mg/kg bw/day)				
	0	3.75	7.5	15.0	30.0
Fetuses examined (*N*)	121	123	114	94	42
Litters examined (*N*)	19	20	18	15	7
Skull					
Os basioccipitale (poorly ossified)					
Fetuses (%)	1.6	4.1	7.9[a]	24.5[a,b,c]	64.3[a,b,c,d]
Litters (%)	10.5	15.0	22.2	40.0[a]	100.0[a,b,c,d]
Os basisphenoid (poorly ossified)					
Fetuses (%)	1.6	6.5	0.9[b]	21.3[a,b,c]	42.9[a,b,c,d]
Litters (%)	10.5	30.0	5.5	33.3[c]	85.7[a,b,c,d]
Os orbitosphenoid (poorly ossified)					
Fetuses (%)	1.6	21.1[a]	0.9[b]	20.2[a,c]	64.3[a,b,c,d]
Litters (%)	10.5	30.0	5.5	40.0[a,c]	85.7[a,b,c,d]
Os frontale (poorly ossified)					
Fetuses (%)	5.0	26.0[a]	6.1[b]	34.4[a,c]	69.0[a,b,c,d]
Litters (%)	26.3	40.0	33.3	46.7	100.0[a,b,c,d]
Os hamulus (poorly ossified)					
Fetuses (%)	0	1.6	0.9	16.0[a,b,c]	14.3[a,b,c]
Litters (%)	0	10.0	5.5	26.7[a]	28.6
Os parietale (poorly ossified)					
Fetuses (%)	5.0	25.2[a]	9.6[b]	37.2[a,c]	90.5[a,b,c,d]
Litters (%)	26.3	35.0	33.3	46.7	100.0[a,b,c,d]
Os lacrimale (poorly ossified)					
Fetuses (%)	2.5	23.6[a]	4.4 [b]	21.3[a,c]	59.5[a,b,c,d]
Litters (%)	15.8	35.0	22.2	40.0	71.4[a,c]
Os nasale (poorly ossified)					
Fetuses (%)	4.1	22.8[a]	6.1[b]	25.5[a,c]	52.4[a,b,c,d]
Litters (%)	21.0	30.0	22.2	40.0	100.0[a,b,c,d]
Os palatinum (wider distance)					
Fetuses (%)	0	27.6[a]	49.1[a,b]	51.1[a,b]	76.2[a,b,c,d]
Litters (%)	0	75.0[a]	100.0[a,b]	80.0[a,c]	100.0[a]
Os praesphenoid (poorly ossified)					
Fetuses (%)	0.8	6.5[a]	6.1[a]	23.4[a,b,c]	38.1[a,b,c]
Litters (%)	5.3	25.0	16.7	40.0[a]	85.7[a,b,c,d]
(unossified)					
Fetuses (%)	0	0	2.6	4.3[a,b]	0
Litters (%)	0	0	5.5	20.0[a,b]	0
Vertebral column					
Sacral vert. (poorly ossified)					
Fetuses (%)	8.3	29.7[a]	45.6[a,b]	64.9[a,b,c]	92.9[a,b,c,d]
Litters (%)	47.4	45.0	88.9[a,b]	86.7[a,b]	100.0[a,b]
Caudal vert. (poorly ossified)					
Fetuses (%)	14.9	30.9 [a]	62.3[a,b]	71.3[a,b]	97.6[a,b,c,d]
Litters (%)	52.6	50.0	94.4[a,b]	86.7[a,b]	100.0[a,b]
Forelimbs					
Clavicle (poorly ossified)					
Fetuses (%)	0	4.1[a]	0.9	13.8[a,b,c]	0[d]
Litters (%)	0	20.0[a]	5.5	26.7[a]	0
Scapula (poorly ossified)					
Fetuses (%)	1.7	20.3[a]	1.8 [b]	14.9[a,c]	4.8[b]
Litters (%)	10.5	30.0	11.1	26.7	14.3
Os humerus (poorly ossified)					
Fetuses (%)	0.8	21.8[a]	1.8[b]	16.0[a,c]	4.8[b]
Litters (%)	5.3	30.0[a]	11.1	26.7	14.3
Os radius (poorly ossified)					
Fetuses (%)	0.8	22.8[a]	6.1[a,b]	16.0[a,c]	7.1[a,b]
Litters (%)	5.3	35.0[a]	33.3[a]	26.7	28.6
Os ulna (poorly ossified)					
Fetuses (%)	0.8	22.8[a]	6.1[a,b]	16.0[a,c]	7.1[a,b]
Litters (%)	5.3	35.0[a]	33.3[a]	26.7	28.6

Fetuses were removed on GD 18. Proportions were analysed by the chi-square test. Differences ($P < 0.05$) are indicated by superscripts as follows: a ≠ 0, b ≠ 3.75 mg/kg, c ≠ 7.5 mg/kg, d ≠ 15 mg/kg.

Table 5
Occurrence of fetal skeleton abnormalities other than poorly ossified/unossified bones and wider distance between palatine bones in the offspring of mice treated orally with triphenyltin hydroxide (TPTH, 0, 3.75, 7.5, 15.0 and 30.0 mg/kg bw/day) on days 6–17 of pregnancy

Treatment	TPTH (mg/kg bw/day)				
	0	3.75	7.5	15.0	30.0
Fetuses examined (*N*)	121	123	114	94	42
Litters examined (*N*)	19	20	18	15	7
Skull					
Os basioccipitale (misshapened)					
Fetuses (%)	4.1	18.7[a]	7.0[b]	11.7[a]	31.0
Litters (%)	15.8	65.0[a]	27.8[b]	40.0	85.7[a,c,d]
(Fused with *os exoccipitale*)					
Fetuses (%)	0	0	0	0	2.4
Litters (%)	0	0	0	0	14.3
Os basisphenoid (misshapened)					
Fetuses (%)	2.5	0.8	1.8	20.2[a,b,c]	31.0[a,b,c]
Litters (%)	15.8	5.0	11.1	40.0[b]	85.7[a,b,c,d]
Os squamosum (additional o.c)					
Fetuses (%)	0	0	0	0	9.5[a,b,c,d]
Litters (%)	0	0	0	0	42.8[a,b,c]
(Fused with *orbitosphenoids*)					
Fetuses (%)	0	0	0 9	0	0
Litters (%)	0	0	5.5	0	0
Os interparietale (misshapened)					
Fetuses (%)	0	0	0	4.3[a,b,c]	7.1[a,b,c]
Litters (%)	0	0	0	26.7[a,b,c]	42.8[a,b,c]
Os supraoccipitale (bipartite)					
Fetuses (%)	0	0	0	9.6[a,b,c]	16.7[a,b,c]
Litters (%)	0	0	0	26.7[a,b,c]	42.8[a,b,c]
Os *lacrimale* (additional o.c)					
Fetuses (%)	0	0	0	0	4.8
Litters (%)	0	0	0	0	28.6
Os palatinum (absent)					
Fetuses (%)	0	0	1.8	0	0
Litters (%)	0	0	5.5	0	0
(Bent)					
Fetuses (%)	0	0	0.9	0	0
Litters (%)	0	0	5.5	0	0
(misshapened)					
Fetuses (%)	0	0	0	0	2.4
Litters (%)	0	0	0	0	14.3
Os praesphenoid (small)					
Fetuses (%)	0	0.8	0	2.1	2.4
Litters (%)	0	5.0	0	6.7	14.3
(misshapened)					
Fetuses (%)	0	0	0	0	16.7[a,b,c,d]
Litters (%)	0	0	0	0	71.4[a,b,c,d]
Processus palatinus des os maxillare (shorter)					
Fetuses (%)	0	0	0.9	0	21.4[a,b,c,d]
Litters (%)	0	0	5.5	0	42.8[a,b,c]
(wider distance)					
Fetuses (%)	0	0	1.8	1.1	16.7[a,b,c,d]
Litters (%)	0	0	5.5	6.7	57.1[a,b,c,d]
Os pterygoides (additional o.c.)					
Fetuses (%)	0	0	0	0	7.1[a,b,c,d]
Litters (%)	0	0	0	0	42.8[a,b,c]
(misshapened)					
Fetuses (%)	0	0	1.8	1.1	11.9[a,b,c,d]
Litters (%)	0	0	5.5	6.7	85.7[a,b,c,d]
Vertebral column					
Atlas (ossified cartilage)					
Fetuses (%)	0	0	0	1.1	4.8
Litters (%)	0	0	0	6.7	28.6
(bent)					
Fetuses (%)	0	0.8	0	0	14.3[a,b,c,d]

(*continued on next page*)

Table 5 (*continued*)

Treatment	TPTH (mg/kg bw/day)				
	0	3.75	7.5	15.0	30.0
Litters (%)	0	5.0	0	0	42.8[a,b,c]
Axis (misshapened)					
Fetuses (%)	0	3.2[a]	0.9	6.4[a,c]	12.0[a,b,c]
Litters (%)	0	15.0	5.5	26.7[a]	57.1[a,b,c]
Cervical vert. (two paired vert absent)					
Fetuses (%)	0	0	0	2.1	0
Litters (%)	0	0	0	6.7	0
Lumbar vert. (addit. two paired vert)					
Fetuses (%)	0	0.81	1.75	0	11.90[a,b,c,d]
Litters (%)	0	5.0	5.5	0	42.85[a,b,c]
(two paired vert absent)					
Fetuses (%)	0	0.81	0	2.13	0
Litters (%)	0	5.0	0	13.30	0
Caudal vert. (absent)					
Fetuses (%)	0	0	0	1.1	0
Litters (%)	0	0	0	6.7	0
Sternum					
Sternebra 1 (fused with sternebra 2)					
Fetuses (%)	0	0	0	2.1	0
Litters (%)	0	0	0	6.7	0
Sternebrae 2 (duplicated)					
Fetuses (%)	0.8	1.6	1.8	1.1	7.1[a]
Litters (%)	5.3	10.0	11.1	6.7	28.6
Sternebra 3 (duplicated)					
Fetuses (%)	0	1.6	4.4[a]	1.1	11.9[a,b,d]
Litters (%)	0	10.0	16.7	6.7	57.1[a,b,c,d]
Sternebra 4 (duplicated)					
Fetuses (%)	0	1.6	4.4[a]	1.1	11.9[a,b,d]
Litters (%)	0	10.0	16.7	6.7	57.1[a,b,c,d]
Sternebra 6 (duplicated)					
Fetuses (%)	0	1.6	1.8	2.1	4.8
Litters (%)	0	10.0	11.1	13.3	28.6
Sternebrae 2, 3, 4 and 5 (fused)					
Fetuses (%)	0	0	0	1.1	0
Litters (%)	0	0	0	6.7	0
Ribs					
(Lumbar, extra, rudimentary)					
Fetuses (%)	1.6	2.4	2.6	4.3	14.3[a,b,c,d]
Litters (%)	10.5	15.0	11.1	6.7	42.8[d]

Fetuses were removed on GD 18. Proportions were analysed by the chi-square test. Differences ($P < 0.05$) are indicated by superscripts as follows: a ≠ 0, b ≠ 3.75 mg/kg, c ≠ 7.5 mg/kg, d ≠ 15 mg/kg.

the skull, vertebral column and forelimbs of the mouse fetus (Table 4). Signs of impaired calcification of fetal skeleton have also been observed in rats (Adeeko et al, 2003) and mice (Tsukamoto et al., 2004) exposed to TBTCl. Along the same line, Tsukamoto et al. (2004) found that, in culture of rat calvarial cells, TBTCl decreased the expression and activity of ALPase and the rate of calcium deposition. The foregoing findings seem to support the view that OTCs impair calcification of fetal skeleton in rats and mice.

In the present study, cleft palate was the most conspicuous anomaly induced by TPTH. A small number of fetuses exposed to TPTH at doses up to 15 mg/kg bw/day exhibited either an incomplete fusion of palate shelves (cleft palate) (1–3.5%), or irregularly shaped palatine rugae. At the highest dose, however, cleft palate appeared in 36.8% of the surviving fetuses and in 71.4% of the litters (Table 2). Skeleton examination revealed that bone anomalies related to palate closure defects—not seen in controls—such as a wider distance between palatine bones and absence of these bones increased in a dose dependent manner and were present in a high proportion (27.6%) of fetuses exposed to the lowest dose of TPTH (Table 3). Our data therefore indicated that the NOAEL for TPTH-produced anomalies of palatine bone is lower than 3.75 mg/kg bw/day. The foregoing NOAEL is lower than the NOAEL for cleft palate (15 mg/kg bw/day), thereby demonstrating that the underlying palatine bone defect is a more sensitive indicator of the adverse effect of this triorganotin compound on palate development.

Cleft palate has been consistently found in rodent in studies of the developmental toxicity of OTCs. Faqi et al. (1997), for instance, reported that, in NMRI mice, oral administration of TBTO on GD 6–17 increased the

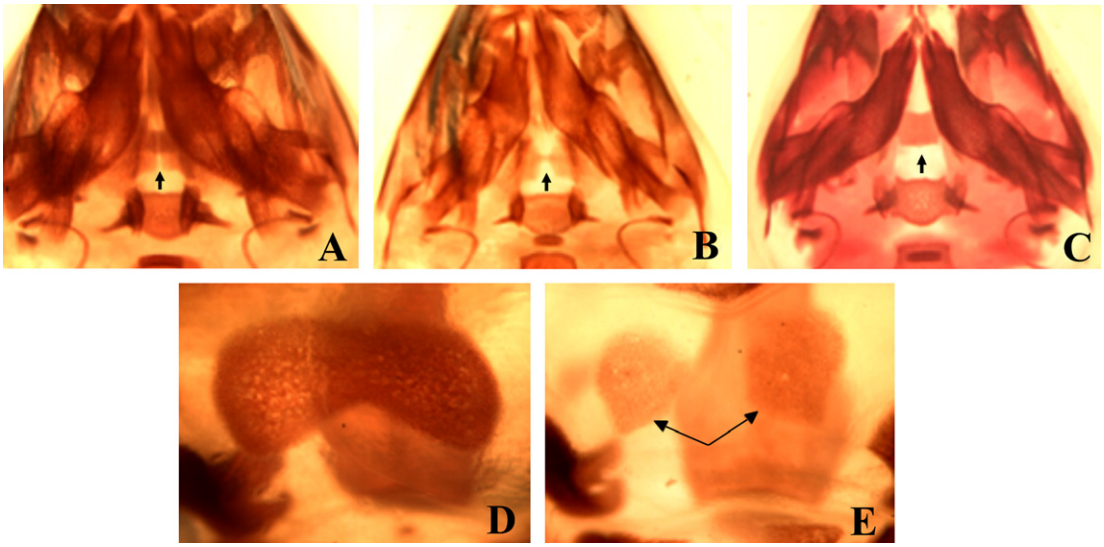

Fig. 1. Fetal skeleton anomalies induced by TPTH in the mouse. (A–C) show skull bones (ventral view) of control (A) and TPTH-treated fetuses (B and C). Arrows indicate a normal (A) and a wider distance between palatine bones (B), as well as the absence of palatine bones (C). (D) (control) shows a dorsal view of the supraoccipital bone of a control fetus and panel (E) (TPTH-exposed fetus) shows a bipartite supraoccipital bone (indicated by arrows). Fetuses (GD 18) were cleared and stained with alizarin red S.

incidence of cleft palate at a dose as high 27 mg/kg bw/day (NOAEL = 13.5 mg/kg bw). According to Faqi et al. (1997), cleft palate was induced by doses of TBTO that were also toxic to the mothers. Davis et al. (1987) also reported that TBTO, at doses overtly toxic to the mothers, increased the occurrence of cleft palate in prenatally-exposed NMRI mice. Cleft palate was also the predominant anomaly in rat fetuses exposed to maternally toxic doses of TBTCl (Ema et al., 1995). Although there have been a number of reports showing that OTCs enhance the occurrence of cleft palate in rodents, whether or not these compounds should be considered as teratogens still remains a controversial matter. Some authors have argued that cleft palate occurs spontaneously in several mouse strains and, also, that its incidence is increased nonspecifically by conditions such as stress and malnutrition (Davis et al., 1987). Another argument against labeling OTCs as teratogens has been the fact that, apparently, they increase the incidence of malformations (cleft palate) only at doses that are also overtly toxic to the mothers. Teratogenic effects observed only under those extreme exposure conditions are generally considered as of minor toxicological relevance. Although the overlapping of embryotoxicity and maternal toxicity indicates that the substance is not selectively toxic to the embryo, it does not necessarily imply that the former arises as a secondary consequence of the latter effect (Iyer et al., 1999). TPTH caused a higher incidence of bone malformations (i.e., including cleft palate) in mice offspring after gestational exposure to a low-dose that did not produce any indication of maternal toxicity. Furthermore, cleft palate seldom occurs spontaneously in Swiss Webster mice from our breeding stock and it was not observed in any control fetus exposed to the same potentially stressful procedure (gavage). Our results therefore indicated that TPTH was teratogenic to Swiss Webster mice. Results presented here also demonstrated that TPTH-induced teratogenicity was apparent at doses that were not overtly toxic to the mothers. As far as the authors are aware, there is no previous study on the developmental toxicity of TPTH in mice.

In conclusion, findings from the present study showed that TPTH, given orally to mice on GDs 6–17, was embryotoxic at doses as low as 3.75 mg/kg bw/day and maternally toxic at doses equal to and higher than 7.5 mg/kg bw/day. It is of note that toxicity was found at doses that are much higher than those to which humans are usually exposed. The general population is exposed primarily through the diet, and market basket studies performed in Japan provided estimates of daily intakes of 4.3 and 2.7 μg (expressed as TPTCl for a 50-kg person) in 1990 and 1997, respectively (Ueno et al., 1999; WHO (World Health Organization) and IPCS (International Program in Chemical Safety), 1999). A similar Finnish study estimated the daily intake of seven organotin compounds (including TPT) as being 2.47 ng per kg bw per day (Rantakokko et al., 2006). Occupational exposure to TPT (and to other OTCs), however, may be substantially higher. Data are needed on the extent of exposures to OTCs in the workplace, including the levels of exposure of women of childbearing age (Ueno et al., 1999; WHO (World Health Organization) and IPCS (International Program in Chemical Safety), 1999, ATSDR (Agency for Toxic Substances and Disease Registry), 2005).

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