Effects of in Utero Tributyltin Chloride Exposure in the Rat on Pregnancy Outcome

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Tributyltin, an organotin, is ubiquitous in the environment. The consumption of contaminated marine species leads to human dietary exposure to this compound. Tributyltin is an endocrine disruptor in many wildlife species and inhibits aromatase in mammal placental and granulosa-like tumor cell lines. We investigated the effects of tributyltin chloride exposure on pregnancy outcome in the Sprague-Dawley rat. Timed pregnant rats were gavaged either with vehicle (olive oil) or tributyltin chloride (0.25, 2.5, 10, or 20 mg/kg) from days 0–19 or 8–19 of gestation. On gestational day 20, dams were sacrificed, and pregnancy outcome was determined. Tributyltin and its metabolites (dibutyltin, monobutyltin) were measured in maternal blood by gas chromatography. Both tributyltin and dibutyltin were present in maternal blood at approximately equal concentrations, whereas monobutyltin contributed minimally to total organotins. Organotin concentrations increased in a dose-dependent pattern in dams, independent of the window of exposure. Tributyltin chloride administration significantly reduced maternal weight gain only at the highest dose (20 mg/kg); a significant increase in post-implantation loss and decreased litter sizes, in addition to decreased fetal weights, was observed in this group. Tributyltin chloride exposure did not result in external malformations, nor was there a change in sex ratios. However, exposure to 0.25, 2.5, or 10 mg/kg tributyltin chloride from gestation days (GD) 0–19 resulted in a significant increase in normalized anogenital distances in male fetuses; exposure from days 8–19 had no effect. There was a dramatic increase in the incidence of low weight (<0.75 of the mean) fetuses after exposure to 20 mg/kg tributyltin chloride. Delayed ossification of the fetal skeleton was observed after in utero exposure to either 10 mg/kg or 20 mg/kg tributyltin chloride. Serum thyroxine and triiodothyronine levels were reduced significantly in dams exposed to 10 and 20 mg/kg tributyltin chloride throughout gestation; in dams treated with tributyltin from GD 8–19, serum thyroxine concentrations, but not triiodothyronine, were significantly decreased at both the 2.5 and 10 mg/kg exposures. Thus, maternal thyroid hormone homeostasis may be important in mediating the developmental toxicity of organotins.

Key Words: organotin; developmental toxicity; reproductive toxicity; fetal ossification; maternal thyroid status.

Organotin compounds result from the addition of organic moieties to inorganic tin and are ubiquitous in the environment. Tributyltin is widely used as an impregnation material in prints and textiles, slime control in paper mills, and a wood preservative, and is also used as a disinfectant in circulating cooling waters. Perhaps the most extensive use of tributyltin is as a defouling agent in paints, for coating structures exposed to the aquatic environment such as ships, oil rigs, pleasure boats, and water intake pipes (Cooney, 1994). Butyltins possess both lipophilic and ionic properties that promote bioaccumulation in lipids and binding to macromolecules upon exposure. Bioconcentration and accumulation of tributyltin in the food chain is well documented; bioconcentration factors of up to 500,000 have been reported in some species (Laughlin, 1996).

Tributyltin enters the human food chain mainly through contaminated marine and freshwater species, from industrial effluents (Snoeij et al., 1987), from domestic use as a wood preservative, by leaching from PVC pipes, and by inhalation and absorption through the skin (Wax and Dockstader, 1995). Occupational exposure to tributyltin occurs primarily during the manufacture and formulation of these compounds, the use of tributyltin as a wood preservative, and the application and removal of tributyltin-containing paints (Corsini et al., 1997). Accidental exposures of humans to organotin compounds have been documented (Saary and House, 2002). In humans, a tolerable daily intake level of 0.25 μg/kg has been proposed based on immunological toxicity (Penninks, 1993). Fatalities from widespread poisoning of humans with organotin occurred...
in France and Algeria in 1954 when Stalinon capsules, containing 15 mg of diethyltin, were used to treat staphylococcal skin infections (cited in Zuckerman, 1958).

The experimental exposure of rodents to organotin compounds produced behavioral and neurological symptoms (Brown et al., 1979) and pancreatic and hepatic toxicities (Merkord et al., 2001). Butyltin compounds impact negatively on the tumor-killing functions of natural killer cells (Whalen et al., 2002). Tributyltin is toxic to the sperm cells and embryos of the Mediterranean sea urchin, Paracentrotus lividus (Novelli et al., 2002). This chemical has been postulated to cause imposex in the mud snail, Ilyanassa obsoleta; imposex consists of the development of male characteristics (mainly a penis and a vas deferens) in female organisms of some gastropod species (Morcillo and Porte, 1999). Although the mechanisms of tributyltin-induced imposex are yet to be fully elucidated, tributyltin is thought to act as a neurotoxin that alters the release of the neupeptide hormone, Penis Morphogenic Factor (Oberdorster and McClellan-Green, 2000). Tributyltin inhibits human aromatase from transfected cells or a granulosa cell–like tumor cell line (Cooke, 2002; Heidrich et al., 2001; Saitoh et al., 2001) and, at noncytotoxic doses, enhances aromatase activity in human placental choriocarcinoma cells (Nakanishi et al., 2002).

There is evidence that exposure to organotins affects mammalian reproduction. Transplacental transfer of organotin was documented in the rat (Noland et al., 1983). In utero exposure of rats to tributyltin chloride reduced maternal weight gain and fetal weights in a dose and phase-specific pattern (Ema et al., 1995); dose-dependent pre- or post-implantation loss (Ema et al., 1995; Harazono et al., 1996, 1998) and fetal toxicity (Itami et al., 1990) were observed.

Humans would normally be exposed to relatively low levels of tributyltin in the diet for long periods, including during pregnancy. Most studies of the developmental toxicity of organotins have assessed the consequences of relatively acute exposures to high doses, and therefore may not provide relevant information with respect to natural exposure paradigms entailing relatively lower doses over a longer duration of exposure. We investigated the consequences of exposure to tributyltin throughout gestation on pregnancy outcome in the Sprague-Dawley rat model. To preclude the pre-implantation embryonic loss that has been demonstrated with tributyltin throughout gestation on pregnancy outcome in the Sprague-Dawley rat model. To preclude the pre-implantation embryonic loss (Harazono et al., 1996), two different windows of exposure to tributyltin chloride were adopted: gestation days (GD) 0–19 and days 8–19. To gain insight as to how windows of exposure to tributyltin chloride were adopted: gestation days (GD) 0–19 and days 8–19. To gain insight as to how the influence of body size as a confounding factor (Gallavan et al., 1999). Fetuses were defined as “low weight” if their weights were less than 0.75 of the mean for their treatment group, and “high weight” if their weights exceeded that of the mean by more than 25%. Two male and two female fetuses were selected randomly from each of five litters in the control, 2.5, 10, and 20 mg/kg tributyltin chloride groups exposed on days 0–19 and the 10 mg/kg tributyltin chloride group exposed from GD 8–19; these fetuses were eviscerated, fixed in 100% ethanol and processed for skeletal staining and evaluation.

MATERIALS AND METHODS

Chemicals. The olive oil vehicle for tributyltin administration was purchased from Aldrich Diagnostics USA (St. Louis, MO) and the tributyltin chloride from Sigma-Aldrich Canada (Oakville, ON). Dibutyltin dibromide (Bu₂SnBr₂, Aldrich Chemical Co., Inc., Milwaukee, WI) and tributyltin chloride (Bu₃SnCl, Gelest Inc., Tullytown, PA) were used as received for the organotin assays. Monobutyltin trichloride (BuSnCl₃, Research Organic/Inorganic Chemical Co., Sun Valley, CA) was distilled prior to use. Tetrasopropyltin (iso-Pro₄Sn), tri-n-propyltin chloride (n-ProSnCl₃) and ethylmagnesium bromide (3M in ether) were used as received from Alfa Aesar (Ward Hill, MA). Bu₂Sn (Gelest Inc., Tullytown, PA) was distilled prior to use. Distilled-in-glass grade solvents (EM Sciences, BDH Inc., Toronto, Canada) and ACS reagent grade chemicals were used for this study. Tropolone was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Lipase (Type VII) and protease (Type XIV) were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and treatment. Male (300–350 g; 8 to 10 weeks old) and female (200–250 g; 9 to 12 weeks old) Sprague-Dawley rats were purchased from Charles River Canada (St. Constant, Quebec) and housed in the McIntyre Medical Building Animal Resource Centre at McGill University with a 14-h light:10-h dark cycle. The animals had free access to food (Purina chow 5012, Mondou Feeds, Montreal, Quebec) and water; all animal handling and care followed the guidelines of the Canadian Council on Animal Care.

Virgin female rats in proestrus were mated overnight with males. Successful mating was indicated by the presence of spermatozoa in the vaginal smear on the following morning (day 0 of pregnancy). Pregnant rats were randomly divided into groups; they were given a daily dose of vehicle (control, n = 25) or tributyltin chloride at 0.25, 2.5, 10 (n = 12/treatment), or 20 mg/kg (n = 13/treatment) by gavage. Tributyltin chloride was administered either from day 0 to 19 of pregnancy or from GD 8 to 19. All tributyltin chloride solutions were prepared fresh daily. Dams were weighed at the initiation of treatment, once every three days thereafter, and on day 20 of pregnancy; the volume of tributyltin administered was adjusted to 5 ml/kg of body weight.

On day 20 of gestation, dams were killed with an overdose of diethyl ether by inhalation. Blood (about 10 ml) was obtained by cardiac puncture; an aliquot (3 ml) was frozen for analysis of organotin levels. The remainder of the blood samples was allowed to clot overnight at 4°C; serum was frozen and stored at –80°C for measurement of thyroid hormones. The ovaries of dams were dissected, and the number of corpora lutea counted. The two-horned uterus was removed and inspected for implantation and resorption sites; pre-implantation loss was calculated as the number of corpora lutea minus the number of implantation sites and post-implantation loss as the number of implantation sites minus the number of fetuses; these values were calculated for each dam. Fetuses were individually weighed and examined for external malformations and the anogenital (A–G) distances were measured; the anogenital distances were normalized with the cube root of the body weight (absolute anogenital distance/cube root of body weight) in order to remove the influence of body size as a confounding factor (Gallavan et al., 1999). Fetuses were defined as “low weight” if their weights were less than 0.75 of the mean for their treatment group, and “high weight” if their weights exceeded that of the mean by more than 25%. Two male and two female fetuses were selected randomly from each of five litters in the control, 2.5, 10, and 20 mg/kg tributyltin chloride groups exposed on days 0–19 and the 10 mg/kg tributyltin chloride group exposed from GD 8–19; these fetuses were eviscerated, fixed in 100% ethanol and processed for skeletal staining and evaluation.

Measurement of organotin concentrations in blood. Blood samples (1 g) were dehydrated overnight at 37°C in 3 ml deionized water (18 meg-ohm resistivity) plus 17 ml EtOH–0.5 M sodium phosphate buffer (pH 8.5) with 50 mg each of lipase and protease, 500 mg Na₂SO₄, 5 mg tropolone, and 5 ml hexane added. Samples were stirred (400 rpm) during the hydrolysis. Reagent blanks were run concurrently with the samples. The samples were cooled to room temperature and the hexane layer collected. Sodium chloride (1.5 g) and 12 M HCl were added until pH 0.5 was reached. The samples were then extracted twice (rotary tumbled at 65 rpm, 20 min) with 10-ml portions of 0.05% tropolone in ether-hexane (1:1). The pooled organic extracts were reduced to 1 ml at 40°C in precalibrated tubes under a nitrogen stream. Tetrahydrofuran (1.5 ml) and ethylmagnesium bromide (0.8 ml) were added to the tropolone extract. The sample tube was then capped under nitrogen.
These operations were conducted inside a nitrogen atmosphere glove box. The samples were then vortexed momentarily, rotary tumbled for 10 min at 25 rpm, and placed in an ice bath. After cooling, the sample volumes were adjusted to 8 ml with prechilled 0.6 M nitric acid, which was initially added drop-wise. Isooctane (2 ml) was added, and the sample tumbled (25 rpm) for 5 min. Centrifugation (2000 rpm, 2 min) hastened phase separation. The organic layer was collected. The aqueous layer was extracted twice with hexane (2 ml, tumbled 25 rpm, 5 min). The pooled organic extracts were then extracted (25 rpm, 5 min) once with 2 ml deionized water (18 meg-ohm resistivity). The water layer was removed and the sample volume reduced to 2 ml at 40°C under a nitrogen stream.

Samples of rat blood (1g) were spiked at two levels (54.3–48.1 ng/g and 543.4–481.2 ng/g) with a mixture containing BuSnCl₃, Bu₂SnBr₂, and Bu₃SnCl prior to hydrolysis. The percentage recovery of each compound was calculated by comparing the mean peak area of the recovered butyltin with the mean peak area of the same compound in a blank clam hydrolysate extract spiked just prior to derivatization. Reagent blanks were run concurrently with each set of samples.

An Agilent model 6890 gas chromatograph (GC) equipped with a model G2350 atomic emission detector (AED) was used for butyltin determination. GC operating conditions were: HP-5 capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Agilent Technologies, Wilmington, DE); carrier gas, He, 2.5 ml/min (constant flow), injector temperature, 250°C; column program, 60°C (0.5 min hold) followed by a linear increase of 8°C/min to 120°C (0.5 min hold), 2°C/min to 150°C (0.5 min hold), and then 8°C/min to 275°C (0.5 min hold). Operating conditions for the AED were: transfer line temperature, 200°C; cavity temperature, 300°C; monitored emission line, 326 nm; cavity pressure, 345 kPa; hydrogen auxiliary gas pressure, 138 kPa; oxygen auxiliary gas pressure, 138 kPa. An auxiliary electronic pressure controller (EPC) regulated the pressure of the AED helium and support gases.

Thyroid hormone measurements in dams. Serum L-thyroxine (T₄) and 3,5,3'-triiodo-L-thyronine (T₃) concentrations were measured in dams using commercially available kits (ICN, Mississauga, Ontario). T₄ level was measured by an ELISA assay, while T₃ was measured by radioimmunoassay. Each sample was measured in duplicate.

Differential staining and evaluation of fetal skeletal development. Ethanol-fixed fetuses were immersed in a water bath (70°C) for 7 s and skinned. Most of the underlying muscles were removed, and the fetuses were placed in 95% ethanol overnight. The following day, the ethanol was discarded and replaced with alcin blue solution (15 mg alcin blue : 80 ml 95% ethanol : 20 ml glacial acetic acid) for 24 h. The alcin blue solution was discarded and replaced with 95% ethanol. After 24 h, 95% ethanol was replaced with an aranzin red S solution (25 mg/l alizarin red S in 1% potassium hydroxide) for 48 h. The stain was drained and replaced with 0.5% potassium hydroxide for 24 h; this was decanted and replaced with a solution consisting of two parts 70% ethanol : 2 parts glycerine : 1 part benzyl alcohol. After 24 h in this 2:2:1 solution, stained skeletons were placed in 1:1 solution (70% ethanol : glycerine) for evaluation and storage. The skull, sternbrae, vertebrae, ribs, pectoral and pelvic girdles, fore and hind limbs were examined.

Statistical analysis. Data were analyzed by one- way or two-way analysis of variance (ANOVA), as appropriate, followed by Tukey, Mann-Whitney rank sum, or t-tests, where significant differences existed.

RESULTS

Organotin Levels in the Blood of Dams

Measurable levels of butyltins were found in blood (Fig. 1) collected from rats of each dosage group and treatment period. Tributyltin and dibutyltin concentration ranges in pregnant animals were similar, from <0.1 or <0.02 ng/g, respectively, in control animals, to 85.5 ng/g or 125.0 ng/g, respectively, in animals exposed to 10 mg/kg tributyltin chloride. The concentrations of both tributyltin and dibutyltin in the blood of dams increased significantly (p < 0.05, one-way ANOVA) with tributyltin chloride dosage (Fig. 1). In contrast, monobutyltin levels were low (<0.01 ng/g in control, to 20.7 ng/g in the group exposed to 10 mg/kg tributyltin).

Pregnancy Outcome

The effects of tributyltin chloride exposure on pregnancy outcome are summarized in Table 1. There was an increase in the number of non-pregnant females only in the highest dose group (20 mg/kg; 4/13), although this increase was not statistically significant; dams in this treatment group, and in the group exposed to 10 mg/kg from GD 8–19, gained significantly less weight during pregnancy than those in other groups. The dams in the 20 mg/kg dose group also appeared to be less physically active. No other apparent signs of maternal toxicity...
**TABLE 1**

**Effects of Tributyltin Chloride on Pregnancy Outcome**

<table>
<thead>
<tr>
<th>Tributyltin chloride (mg/kg)</th>
<th>Day 0–19</th>
<th>Day 8–19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>No. females mated</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>No. of dams pregnant</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>No. implantation sites</td>
<td>14.8</td>
<td>14.8</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>No. dead fetuses</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>0.52</td>
<td>0.51</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>3.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Male</td>
<td>2.69</td>
<td>2.84</td>
</tr>
<tr>
<td>Female</td>
<td>1.41</td>
<td>1.42</td>
</tr>
<tr>
<td>Sex ratio</td>
<td>159/167</td>
<td>101/81</td>
</tr>
</tbody>
</table>

*Significantly different from control (p < 0.05); **(p < 0.005). Two-way ANOVA followed by Tukey, Mann-Whitney rank sum, or t-test. Numbers are means ± SEM.

were observed in any of the animals exposed to tributyltin chloride.

Although the incidence of preimplantation loss was not increased in any of the tributyltin chloride treatment groups, there was a significant increase in postimplantation loss among dams exposed to 20 mg/kg tributyltin chloride from GD 0–19. As expected, litter size was also decreased. The highest number of dead fetuses (three) was found in this treatment group; however, this incidence was not statistically different from control. Three dead fetuses were also found in the litters of dams treated with 10 mg/kg tributyltin chloride (from GD 0–19 and 8–19), but there were none in any of the other treatment groups. Litters exposed to either 10 or 20 mg/kg tributyltin chloride had enlarged placentas. The mean litter weights of male and female fetuses were decreased by 33% to 43%, respectively, in the group exposed to 20 mg/kg tributyltin chloride. The incidence of “low weight” fetuses (≤0.75 of the mean) in the 20 mg/kg tributyltin chloride treatment group increased dramatically to attain 54.4%, greatly exceeding the incidence in control litters (1.5%). The incidence of “high weight” fetuses (≥125% of the mean) was increased significantly above control (0.3%) only in the GD 8–19 group exposed to 2.5 mg/kg tributyltin chloride (11.5%). The anogenital distances in male fetuses were increased significantly compared to control after in utero exposure to 0.25, 2.5, or 10 mg/kg tributyltin chloride from GD 0–19; the anogenital distances were not affected either by exposure to these doses from GD 8–19 or by exposure to the highest tributyltin dose (20 mg/kg) from GD 0–19. Exposure of female fetuses to tributyltin chloride also had no significant effect on anogenital distances. Sex ratios, as assessed by anogenital distances, were not altered by in utero exposure to tributyltin, nor did exposure to tributyltin induce external malformations in the fetuses.

**Skeletal Development**

Variations in ossification of the sternae and an example of sternoschisis are illustrated in Figure 2. The incidence of bipartite sternae, presenting as ossified loci in the sternum, was significantly higher in fetuses of dams gavaged with 10 or 20 mg/kg tributyltin chloride from days 0–19 compared to controls (Fig. 3). Although a similar trend was shown with exposure to 10 mg/kg from days 8–19, this was not significant (Table 2). Reduced ossification was also seen in the pelvic girdle, skull, and limbs of fetuses of dams exposed to 20 mg/kg tributyltin chloride, however, this did not reach statistical significance. Two fetuses in this group and in the 10 mg/kg tributyltin chloride group had a misaligned or split sternum (sternoschisis) (Fig. 2D). No variations in skeletal ossification or skeletal malformations were observed in the control or 2.5 mg/kg tributyltin chloride treatment groups (Table 2).

**Effects of Tributyltin Exposure on Thyroxine and Triiodothyronine Levels in Dams**

Serum levels of $T_3$ in the control dams ($n = 7$) were in the normal range detected using a similar methodology (Christenson et al., 1996) (Fig. 4). Serum $T_3$ levels were significantly
decreased in the dams exposed to 10 or 20 mg/kg tributyltin chloride from GD 0–19, whereas exposure to 0.25 or 2.5 mg/kg tributyltin chloride had no significant effect. Likewise, serum T₃ levels in tributyltin chloride-treated dams were significantly lower than control levels after exposure to the 10 or 20 mg/kg doses from GD 0–19. After exposure to tributyltin chloride from days 8–19 of gestation, serum T₄ levels were significantly decreased in both the 2.5 and 10 mg/kg groups; serum T₃ levels were not significantly different from controls. Thus, exposure to 10 or 20 mg/kg tributyltin chloride during pregnancy dramatically affected circulating thyroid hormone levels.

**DISCUSSION**

Tributyltin, dibutyltin, and monobutyltin were measured in the blood of pregnant rats gavaged with varying doses of tributyltin chloride. Data from the present study indicate that the levels of butyltins in dams’ blood were dependent on the dose. Dealkylation of tributyltin to dibutyltin resulted in similar levels of the two compounds in the blood; however, monobutyltin constituted only a very small percentage (less than 5%) of the total organotin present, suggesting a low in situ conversion of dibutyltin to monobutyltin.

Tributyltin exposure reduced maternal weight gain in a complex dose and treatment duration-dependent pattern. When compared to dams treated with lower doses, dams exposed to 10 mg/kg tributyltin chloride gained significantly less weight when treatment was initiated on day 8 of gestation. This reduction in weight gain was not dependent solely on tributyl-
tin chloride exposure, because blood levels of organotin were not significantly different from those in rats exposed over the entire period from GD 0 to 19. Stress-related animal handling (Marti et al., 1994) could not be solely responsible for this discrepancy, since weight gain in the rats gavaged with low doses of tributyltin chloride during the same window did not differ from controls. One possibility is that the reduction in weight gain may be attributed to a decrease in the serum level of thyroxine (Versloot et al., 1998). The circulating T4 concentrations in dams gavaged with 10 mg/kg tributyltin chloride from days 8–19 were approximately half of those in dams gavaged with the same dose from days 0–19 (8.1 and 16.8 ng/ml, respectively).

Although preimplantation loss was not significantly different between control and treated dams, postimplantation loss was significantly higher at the highest tributyltin chloride dose (20 mg/kg). High doses of tributyltin (32.5 mg/kg on gestational days 0 to 3; 16.3 mg/kg and upwards when administered from days 4 to 7) were previously reported to induce postimplantation loss (Harazono et al., 1998). Interestingly, the few dead fetuses observed in this study occurred in the litters from dams gavaged with the high doses of tributyltin chloride. The process of implantation appears to be less susceptible to organotin than mechanisms responsible for the maintenance of the implanted embryo.

Although fetal weights were reduced in litters exposed to the highest dose of tributyltin chloride (20 mg/kg), the mean placental weights were increased, both in this group and in the litters exposed to 10 mg/kg tributyltin chloride from gestational days 0–19 or 8–19. Thus, it is clear that any placental enlargement induced by exposure to tributyltin is not associated with enhanced fetal growth, but rather with reduced fetal weights and even embryo or fetal death. This is interesting because in

### TABLE 2

Effects of in Utero Exposure to Tributyltin Chloride on Skeletal Development in Fetuses

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>2.5 mg/kg GD 0–19</th>
<th>10 mg/kg GD 0–19</th>
<th>10 mg/kg GD 8–19</th>
<th>20 mg/kg GD 0–19</th>
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<tbody>
<tr>
<td>n = 16</td>
<td>n = 24</td>
<td>n = 20</td>
<td>n = 18</td>
<td>n = 23</td>
<td></td>
</tr>
<tr>
<td>Variations in skeletal development</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sternum:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unossified sternaebae</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Ossification of 1st sternebra only</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bipartite sternebrae</td>
<td>0</td>
<td>0</td>
<td>19*</td>
<td>10</td>
<td>16*</td>
</tr>
<tr>
<td>Misalignment of sternum</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pelvic girdle:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced ossification</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Fore and hind feet:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Metacarpals and metatarsals not ossified</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Skull</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supraocciptals (not fused)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Skeletal malformations</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sternoschisis</td>
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</table>

*Significantly different from control (p < 0.01). Fisher Exact Test.

![Graph showing serum thyroxine (top) and triiodothyronine (bottom) concentrations in gestational day 20 dams gavaged with vehicle (olive oil) or tributyltin chloride from gestational days 0–19 (0.25, 2.5, 10, or 20 mg/kg) or 8–19 (0.25, 2.5, 10 mg/kg). Values represent means ± SEM. *Significantly different from control p < 0.01.](https://academic.oup.com/toxsci/article-abstract/74/2/407/1716351)
In summary, fetal weight correlates positively with placental weight (Heasman et al., 1999). An increase in placental weight induced by in utero exposure to tributyltin has been documented previously (Itami et al., 1990), but the mechanisms underlying this effect are not known.

While, in gastropod species, tributyltin is a known endocrine disruptor implicated in the development of imposex, in utero exposure of rats to tributyltin chloride did not affect the fetal sex ratio (current study; Ema et al., 1995; Harazono et al., 1996, 1998). These data suggest that, in the fetal rat, tributyltin chloride exposure does not alter sex differentiation or proper formation of the external genitalia, even when exposure precedes and is maintained throughout organogenesis. A change in fetal sex ratio, characterized by a disproportionately higher number of male fetuses, was reported when dams were gavaged with a single high dose (100 or 200 mg/kg) of tributyltin on gestational days 9 or 7, respectively (Ema et al., 1997). In the same report, post-implantation loss was significantly increased, suggesting that tributyltin was selectively embryolethal to female fetuses at high doses. The chronic exposure during pregnancy to tributyltin at lower doses does not suggest such a sex-dependent bias in embryolethality.

In utero exposure to tributyltin chloride selectively affected male fetal anogenital distances. Interestingly, anogenital distances were increased significantly in male fetuses exposed to tributyltin chloride (0.25, 2.5, or 10 mg/kg/day) from gestational days 0 to 19, but not from days 8–19. This finding suggests that there is a critical window of exposure prior to the development of the perineum. Furthermore, a dose-dependent response was not observed despite a log difference in dose. It is likely that there was no effect on male anogenital distance in the highest dose group (20 mg/kg) because of the severe decreases in fetal size. In a previous report, an insignificant increase in anogenital distance was shown in postnatal day 1 in male rats exposed to tributyltin chloride (125 ppm) during gestation and lactation (Oamura et al., 2001). In contrast, in utero and lactational exposures (5, 25, or 125 ppm) to tributyltin chloride significantly increased anogenital distance in postnatal day 1 female fetuses (Ogata et al., 2001). While the mechanisms underlying these findings are not known, it is unlikely that discrepancies in the effects of tributyltin exposure on anogenital distances between previous reports and this study are due to the timing of observation (fetal versus neonatal).

Tributyltin exposure did not result in external malformations in the present study. This finding is consistent with previous reports in which in utero exposure to tributyltin at maternal doses as high as 65.1 mg/kg did not induce malformations (Harazono et al., 1996, 1998). Very high single doses of tributyltin (100 or 200 mg/kg) have been reported to increase the incidence of external malformations, comprising mainly of cleft palate; the critical periods for these fetal malformations were GD 8 and 11–14 (Ema et al., 1997), or days 13 to 15 (53.8 and 107.6 mg/kg tributyltin, respectively) (Ema et al., 1996).

In the present study, exposure to doses of tributyltin chloride of 10 or 20 mg/kg from GD 0 to 19 was associated with reduced ossification in the fetuses; furthermore, sternoschisis (split sternum) was observed in two fetuses in the 20 mg/kg tributyltin chloride treatment group. Embryonic growth retardation may underlie reduced ossification among the fetuses exposed to 20 mg/kg tributyltin chloride but is unlikely to account for misaligned sternebrae or sternoschisis. In addition, reduced ossification of the sternebrae was observed among the fetuses exposed to 10 mg/kg tributyltin chloride, for which weights were in the normal range. These data indicate that mechanisms other than low fetal weight, presumably directly related to tributyltin chloride, may contribute to this feature. In this context, it is especially interesting that exposure to 10 or 20 mg/kg tributyltin chloride from GD 0–19 significantly reduced serum thyroxine and triiodothyronine levels.

Thyroid hormones regulate metabolism and body weight in mammals; thyroidectomy and the ensuing hypothyroidism reduce maternal weight gain during pregnancy (Versloot et al., 1998). Exposure of young male rats to bis(tri-n-butyltin)oxide (tributyltino) for six weeks reduced serum thyroxine and thyrotropin concentrations (Krajnc et al., 1984). In contrast, chronic exposure of male rats to tributyltin had no effect on serum thyrotropin levels, although the free thyroxine : thyrotropin ratio was decreased (Wester et al., 1990). The mechanisms involved in the reduction in circulating thyroid hormone levels observed in the present study are unknown. Tributyltin may be toxic to the thyroid gland and decrease the synthesis of thyroid hormones. Tributyltin was cytotoxic to cortical astrocytes (Rohl et al., 2001), thymocytes (Gennari et al., 2002), and natural killer cells (Whalen et al., 2002) and caused thymic atrophy in vivo (Snoeij et al., 1985, 1988). Tributyltin may enhance the biliary excretion of T₄, since rats chronically exposed to tributyltin compounds developed inflammatory biliary duct lesions (Snoeij et al., 1987). Alternatively, the levels of the plasma thyroid hormone transport protein may be reduced by tributyltin, due to its inhibition of protein synthesis, thereby reducing circulating levels of the hormone.

The delayed appearance of ossification centers is a frequent finding in newborns with congenital hypothyroidism (Greenberg et al., 1974), and reduced radiological ossification centers were found in the fetuses of dams thyroidectomized prior to mating (Gil-Garay et al., 1991). Thus, maternal hypothyroidism may result in hypothyroid fetuses with delayed skeletal ossification. It is likely that the tributyltin-induced disturbances in maternal thyroid hormone homeostasis contribute to the reduction in fetal skeletal ossification that was observed.

Adverse pregnancy outcomes were observed after tributyltin exposure in the high dose exposure groups. While it is unlikely that dietary or even occupational exposures would put humans at high risk, women who are hypothyroid may represent a sensitive population.
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