Table 3
Body weight after weaning in offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of male offspring	63	61	60	55	60
Body weight of male offspring (g)[a]					
Postnatal day 28	101 ± 7	102 ± 8	98 ± 6	100 ± 6	92 ± 9**
Postnatal day 35	161 ± 12	163 ± 13	157 ± 10	160 ± 10	151 ± 13**
Postnatal day 42	225 ± 15	230 ± 16	221 ± 14	225 ± 14	217 ± 15**
Postnatal day 49	289 ± 20	297 ± 20	285 ± 18	289 ± 18	281 ± 17*
Postnatal day 56	346 ± 24	356 ± 24	342 ± 22	345 ± 24	336 ± 22*
Postnatal day 63	390 ± 27	400 ± 27	384 ± 26	388 ± 27	379 ± 25
Postnatal day 70	424 ± 32	436 ± 30	418 ± 30	422 ± 32	414 ± 33
No. of female offspring	63	62	58	57	63
Body weight of female offspring (g)[a]					
Postnatal day 28	90 ± 7	93 ± 6	90 ± 5	90 ± 5	85 ± 7**
Postnatal day 35	135 ± 9	138 ± 11	135 ± 9	135 ± 7	130 ± 10*
Postnatal day 42	171 ± 13	175 ± 13	172 ± 12	171 ± 10	168 ± 13
Postnatal day 49	196 ± 14	204 ± 15*	200 ± 15	199 ± 12	196 ± 15
Postnatal day 56	222 ± 17	230 ± 17*	226 ± 17	222 ± 14	221 ± 18
Postnatal day 63	239 ± 19	248 ± 19*	246 ± 22	243 ± 17	241 ± 19
Postnatal day 70	254 ± 20	264 ± 22*	262 ± 22	258 ± 18	257 ± 21

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

Table 4
Physical development in offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No of litters examined	22	21	20	19	21
Age at pinna unfolding (days)[a]					
Male	2.9 ± 0.6	3.0 ± 0.5	3.0 ± 0.5	2.9 ± 0.7	3.1 ± 0.5
Female	2.8 ± 0.5	2.9 ± 0.5	3.0 ± 0.5	3.1 ± 0.6	3.1 ± 0.6
Body weight at pinna unfolding (g)[a]					
Male	8.6 ± 0.7	9.3 ± 1.3	9.0 ± 1.0	8.9 ± 0.5	8.8 ± 1.0
Female	8.1 ± 0.8	8.8 ± 1.0	8.6 ± 1.0	8.5 ± 0.5	8.4 ± 0.9
Age at fur appearance (days)[a]					
Male	9.0 ± 0.4	9.0 ± 0.4	9.0 ± 0.6	9.1 ± 0.5	9.0 ± 0.5
Female	9.1 ± 0.4	9.1 ± 0.4	9.2 ± 0.6	9.2 ± 0.5	9.1 ± 0.6
Body weight at fur appearance (g)[a]					
Male	22.2 ± 1.5	23.2 ± 1.8	21.9 ± 1.8	22.5 ± 1.6	20.9 ± 2.2*
Female	21.5 ± 1.5	22.3 ± 1.7	21.2 ± 1.8	22.0 ± 1.5	20.5 ± 2.3
Age at incisor eruption (days)[a]					
Male	10.0 ± 0.8	10.1 ± 0.5	10.1 ± 0.5	10.1 ± 0.7	10.1 ± 0.9
Female	10.0 ± 0.6	10.0 ± 0.7	10.1 ± 0.4	9.9 ± 0.6	10.0 ± 1.0
Body weight at incisor eruption (g)[a]					
Male	24.6 ± 2.2	25.7 ± 1.6	24.4 ± 2.5	25.0 ± 1.8	23.1 ± 3.2
Female	23.5 ± 1.6	24.3 ± 1.7	23.6 ± 2.5	24.0 ± 1.3	22.4 ± 3.0
Age at eye opening (days)[a]					
Male	15.3 ± 0.5	15.2 ± 0.7	15.3 ± 0.5	15.4 ± 0.7	15.3 ± 0.6
Female	15.4 ± 0.5	15.2 ± 0.5	15.2 ± 0.5	15.3 ± 0.6	15.2 ± 0.6
Body weight at eye opening (g)[a]					
Male	37.9 ± 1.6	39.4 ± 2.4	37.7 ± 2.6	38.5 ± 2.0	35.1 ± 3.1*
Female	36.9 ± 1.6	37.6 ± 2.0	36.0 ± 2.1	37.3 ± 1.7	34.1 ± 3.1*

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.

Table 5
Sexual maturation of offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
Male preputial separation					
No. of male pups examined	22	21	20	18	21
Age (days)[a]	40.5 ± 1.5	40.5 ± 1.2	40.9 ± 1.3	40.3 ± 1.1	41.0 ± 1.3
Body weight (g)[a]	206 ± 32	205 ± 24	207 ± 22	205 ± 25	202 ± 28
Female vaginal opening					
No. of female pups examined	22	21	20	19	21
Age (days)[a]	33.3 ± 1.9	33.6 ± 1.6	33.9 ± 1.4	32.8 ± 0.9	33.4 ± 2.2
Body weight (g)[a]	124 ± 10	130 ± 14	127 ± 9	121 ± 9	121 ± 16

[a] Values are given as the mean ± S.D.

The rate of successfully conditioned responses for every 10 min test period on PNDs 60–67 is shown in Fig. 5. No significant changes were found in males and females of any PS80-treated groups when conditioned avoidance responses were determined on PNDs 60–67.

3.6. Necropsy and histopathology in offspring

There were no compound-related gross lesions in males and females at necropsy on PND 22. Table 6 shows absolute and relative organ weights on postnatal day 22 in male and female offspring. There were no significant differences in absolute and relative weights of the brain, liver, spleen, adrenal or kidney in male and female pups between control and PS80-treated groups.

No histopathological changes in the cerebrum, cerebellum, medulla oblongata, pons, spinal cord in the thoracic and lumbar regions, and sciatic nerve were noted in 22-day-old males and females of the control and 7.5% groups (data not shown).

No compound-related gross lesions were found in males and females at necropsy on PND 70 and on PNDs 103–126. There were no significant differences in the absolute and relative weights of the brain, liver, spleen, adrenal or kidney in 70-day-old male and female pups between control and PS80-treated groups. Although slight mononuclear cell infiltration in the choroid plexus was observed in the cerebrum in one male in the control group, no other histopathological changes in the cerebrum, cerebellum, medulla oblongata, pons, spinal cord in the thoracic and lumbar regions, and sciatic nerve were found in 70-day-old males and females of control and 7.5% groups (data not shown).

4. Discussion

A developmental neurotoxicity study was performed to evaluate the potential functional and morphological effects of PS80 on the developing nervous system of offspring of rats given PS80 during pregnancy and lactation. This study was designed to assess both continuous parameters, such as body weight and food and water consumption, and parameters at specific times, pre-weaning, adolescence and young adult periods, such as physical development, reflex ontogeny, sexual maturation, motor activity, motor and sensory function, learning, and pathological findings, and to further assess reproductive and developmental endpoints.

In the present study, loose stool during lactation was observed in many dams given drinking water containing PS80 at 7.5%. In a previous 2-year breeding study, diarrhea was observed in rats

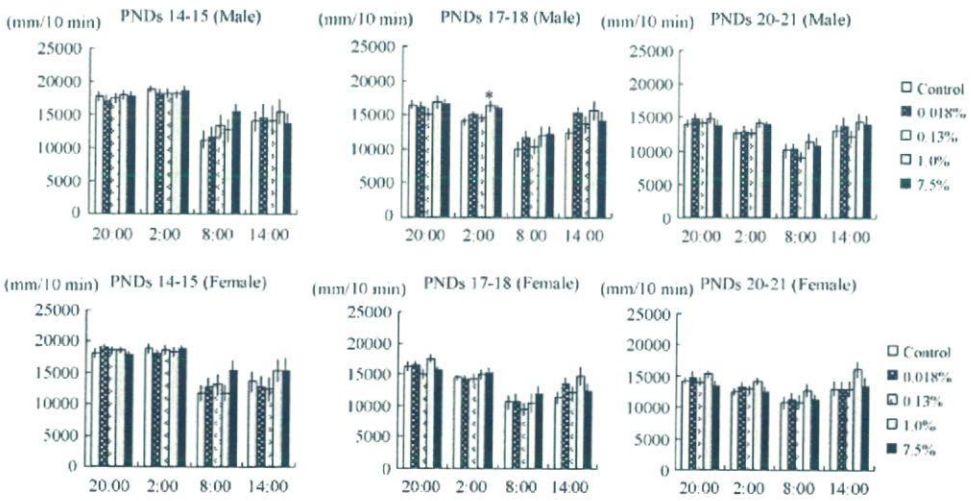

Fig. 3. Locomotor activity in pre-weaning offspring of rats given polysorbate 80 during pregnancy and lactation. Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

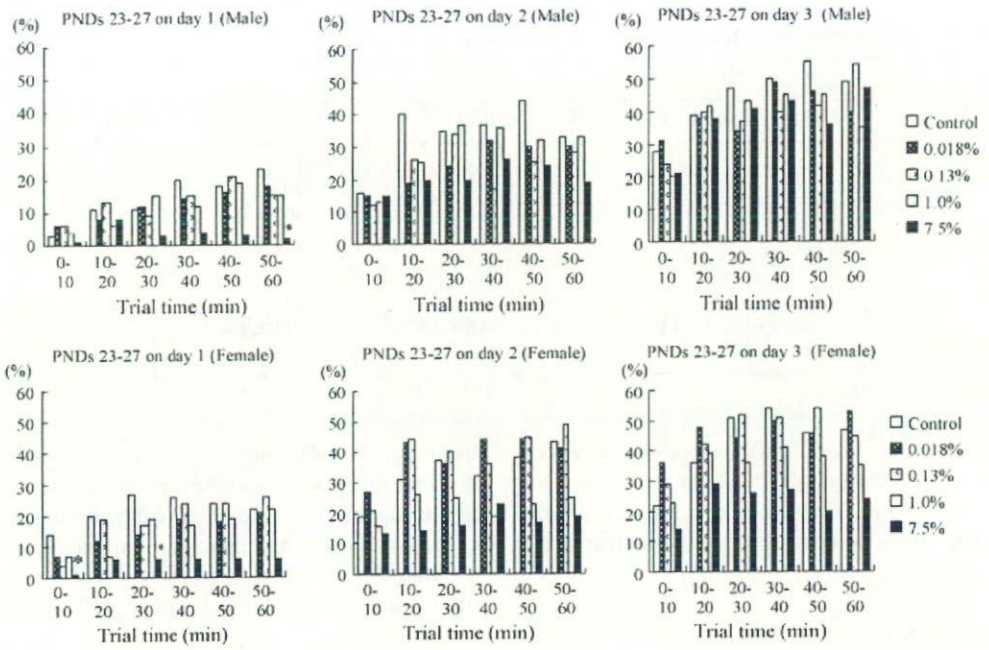

Fig. 4. The rate of successful responses in conditioned avoidance test on postnatal days 23–27 in offspring of rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

fed a diet containing PS80 at 10% and higher [12]. The diarrhea observed in feeding studies with polysorbates seems to result from having high concentrations of the unabsorbed poloxyethylene sorbitan moiety within the intestinal lumen [13,14]. The decrease in body weight and body weight gain accompanied by decreased food and water consumption was also noted at 7.5%; however, no significant findings in clinical observations, body weight, body weight gain, or food and water consumption were detected at 1.0% and below. Reduced water consumption may be due to slight characteristic scent and unpleasant and slightly bitter taste of PS80 [15]. However, lower water consumption was noted only on 2 days at the highest dose. These findings do not indicate poor palatability of PS80 in water and dose–dependent taste aversion. PS80 seems to be dosed successfully by this route. Dilatation of the cecum was also observed at 7.5%. Although increased relative weight of the kidney was

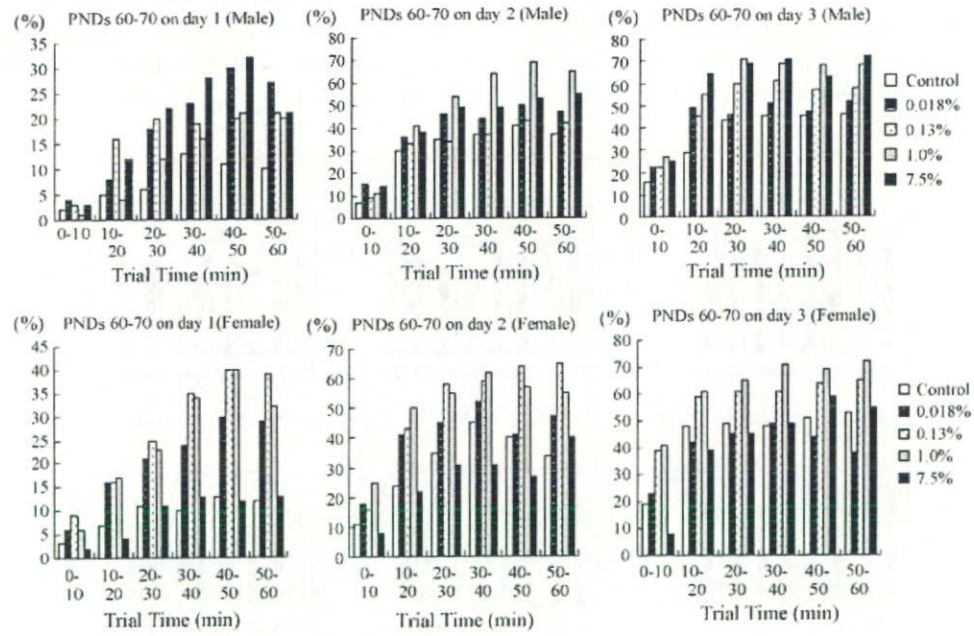

Fig. 5. The rate of successful responses in conditioned avoidance test on postnatal days 60–70 in offspring of rats given polysorbate 80 during pregnancy and lactation.

Table 6
Absolute and relative organ weights on postnatal day 22 in male and female offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of male pups	11	11	11	9	10
Body weight (g)[a]	58.9 ± 6.8	61.1 ± 5.1	60.3 ± 4.2	62.2 ± 3.6	55.0 ± 6.3
Brain (g)[a]	1.49 ± 0.05[b]	1.55 ± 0.06	1.53 ± 0.06	1.53 ± 0.05	1.49 ± 0.07
	2.56 ± 0.29[c]	2.55 ± 0.18	2.54 ± 0.18	2.47 ± 0.17	2.75 ± 0.33
Liver (g)[a]	2.45 ± 0.43	2.38 ± 0.38	2.25 ± 0.23	2.44 ± 0.21	2.21 ± 0.38
	4.18 ± 0.77[c]	3.88 ± 0.37	3.74 ± 0.29	3.92 ± 0.23	3.99 ± 0.33
Spleen (mg)[a]	266 ± 57	308 ± 65	302 ± 52	290 ± 32	274 ± 45
	449 ± 59[d]	502 ± 91	500 ± 66	466 ± 35	495 ± 37
Adrenal (mg)[a]	21 ± 4	23 ± 3	22 ± 5	22 ± 4	21 ± 4
	35.5 ± 5.7[d]	38.0 ± 4.8	36.8 ± 8.1	35.2 ± 6.6	38.9 ± 9.5
Kidney (mg)[a]	720 ± 97	746 ± 80	725 ± 61	738 ± 49	666 ± 53
	1222 ± 84[d]	1219 ± 70	1202 ± 45	1187 ± 76	1218 ± 72
No. of female pups	11	11	11	9	10
Body weight (g)[a]	55.2 ± 6.3	56.5 ± 6.0	55.8 ± 3.9	57.5 ± 4.3	52.4 ± 5.6
Brain (g)[a]	1.45 ± 0.05[b]	1.47 ± 0.06	1.45 ± 0.06	1.46 ± 0.04	1.43 ± 0.08
	2.65 ± 0.28[c]	2.63 ± 0.29	2.61 ± 0.21	2.54 ± 0.16	2.75 ± 0.25
Liver (g)[a]	2.09 ± 0.38	2.15 ± 0.34	2.21 ± 0.43	2.23 ± 0.21	2.07 ± 0.27
	3.77 ± 0.35[c]	3.78 ± 0.26	3.99 ± 0.86	3.87 ± 0.14	3.95 ± 0.25
Spleen (mg)[a]	263 ± 44	295 ± 56	266 ± 30	265 ± 51	262 ± 36
	479 ± 80[d]	519 ± 75	477 ± 40	458 ± 64	501 ± 50
Adrenal (mg)[a]	23 ± 4	21 ± 4	22 ± 5	22 ± 6	19 ± 2
	41.5 ± 6.6[d]	37.7 ± 7.2	39.0 ± 8.8	38.6 ± 10.1	36.4 ± 5.5
Kidney (mg)[a]	717 ± 90	724 ± 72	711 ± 65	726 ± 66	676 ± 52
	1299 ± 92[d]	1282 ± 57	1277 ± 99	1262 ± 82	1294 ± 74

[a] Values are expressed as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g)/100 g body weight.
[d] Relative organ weight = organ weight (mg)/100 g body weight.

observed at 7.5%, this change was not thought to have toxicological meaning because of no changes in the gross pathology or in absolute weight. These findings indicate that the NOAEL for general toxicity in maternal rats was 1.0% (1.864 ml/kg bw/day).

In previous studies, the reproductive and developmental effects of PS80 were investigated in rats and mice given relatively high doses of PS80. No adverse effects on reproductive and developmental outcome were noted in rats fed a diet containing PS80 at 2% through three generations [16]. Fertility and offspring survival were diminished in a 2-year breeding study using rats fed a diet containing PS80 at 20%, but not at 5 or 10% [17]. A prenatal developmental toxicity study revealed no clear adverse effects in dams and fetuses of rats given PS80 at 500 and 5000 mg/kg bw/day by gavage on days 6–15 of pregnancy [18]. Administration of PS80 at 2500 mg/kg bw/day by gavage on days 8–12 of pregnancy caused no adverse effects on dams or offspring of mice [19]. In these previous studies, no detailed information on reproductive and developmental parameters was reported. Although a few females showed reproductive difficulties in PS80-treated groups in the present study, necropsy of the reproductive organs revealed no evidence of reproductive failure in these rats. No changes in the fecundity index, gestation length or gestation index were noted in any PS80-treated groups; however, the number of pups born was significantly decreased at 7.5%. One possible explanation for this decrease may be the slight decrease in the number of implantations. It is not known whether the decreased number of implantations was attributable to a decreased number of corpora lutea or an increase of number of pre-implantation embryonic loss, because the dams were sacrificed 21 days after delivery and the number of corpora lutea was not determined in the present study. No information on the adverse effects of PS80 on formation of the corpora lutea, implantation process and pre-implantation embryonic loss is available in previous reproductive and developmental toxicity studies of PS80 [16–19]. In the present study, acaudate and anal atresia were found in one pup at 0.018%; however, the incidence of malformations was very low, not dose-related and not significantly different from that in the control group. The external malformations observed in the present study were of the types that occur spontaneously among control rat fetuses reported in the literature [20–23]; therefore, it seems unlikely that the morphological changes in pups observed in the present

study indicate a teratogenic response. Maternal administration of PS80 at 7.5% caused the low body weight of male and female offspring during the pre-weaning period, and these changes were accompanied with the decreased weight of maternal rats. Body weight of offspring during the post-weaning period was also lower at 7.5%. The effect on pup weight showed up later but may actually have been present at birth in the 7.5% group, because the smaller number of litter mates per dam might have heavier body weight of pups in this group. These findings indicate that the dose level of 7.5% used in this study was potent enough to have adverse effects on growth of the offspring.

In the present study, the body weights at the age of completed fur appearance in male pups and eye opening in male and female pups were reduced at 7.5%; however, no significant changes were found in the age of completed these developmental landmarks. No changes were detected in reflex ontogeny and sensory function in male and female offspring given PS80. In addition, PS80 did not cause any changes in indicators of the onset of sexual maturity. It seems unlikely that PS80 affects the functional development and sexual maturation of offspring.

In the previous developmental neurotoxicity study of PS80 [6], daily locomotor activity and diurnal locomotor activity were increased in male offspring of rats given PS80 at 0.125% in their drinking water. The locomotor activity of male pups was determined during the pre-weaning period using a cage consisting of two sections, a home cage section with exploration holes that allowed movement of the pups back and forth to a second section, the exploration cage while restricting the movement of the dam to the home cage [24]. The OECD Draft Test Guideline 426 Developmental Neurotoxicity Study [7] noted that motor activity should be monitored during the pre-, peri- and post-weaning periods, including the young adult period, by an automated activity recording apparatus and that the animals should be tested individually. Monitoring nocturnal activity in rodents is important for toxicological studies [25,26], because rodents are more active during the nocturnal period [27–29] and neurotoxicants may be more effective during this period. In the present study, the motor activity of pups was individually determined during diurnal and nocturnal periods in the pre-, and peri-weaning, and young adult periods using automated activity recording apparatus. Although higher activity was detected in male pups at 2:00 on PND 18 in the 1.0% group, this change was discontinuous, inconsistent across sexes and not dose-related; therefore, this change was not thought to be due to the administration of PS80 and had no toxicological significance. No changes in locomotor activity were observed in PS80-treated pups of both sexes during other test periods. These findings indicate that PS80 is ineffective on locomotor activity in male and female pups of rats fed this compound during pregnancy and lactation.

Although a decreased rate of successfully conditioned avoidance responses was found at 7.5% in males and females on PNDs 23–27, no changes were found in both sexes of any PS80-treated groups on PNDs 60–67. It is likely that PS80 at 7.5% caused a transient suppression of conditioned avoidance responses in pups of both sexes. However, necropsy and histopathological examinations, including the nervous system, revealed no evidence of developmental disorders in pups on PNDs 22 and 70. The magnitude of decrease in body weight of pups was more pronounced during the younger stage than the older stage. It is noted that light body weight mice performed worse than heavy body weight mice in a learning task [30]. The possibility remains that the lowered conditioned avoidance response determined on PNDs 23–27 may be due to the reduced body weight of pups.

As for growth retardation of offspring, it is known that there are strong positive correlations between developmental landmark parameters and the weight of pups [31] and the best indicator of physical development is body weight [32] in experimental animal studies. Neurobehavioral teratology studies of some organic solvents have shown that decreased birth weight and functional impairment can be caused by the same chemicals at the same dosage levels [33]. In humans, the association of intrauterine growth retardation has been amply demonstrated with respect to neurological dysfunction [34]. Furthermore, human infants who show evidence of growth retardation have a 33–50% likelihood of having a learning disability [33,35]. These reports indicate that developmental neurotoxicity parameters are often associated with growth retardation, which is also an important parameter in developmental neurotoxicity studies. In the present study, transient decrease of successful responses in the conditioned avoidance test and reduced body weight were found at 7.5%, but no neuropathological changes were detected. The exposure of pups during the lactation period may be partly indirect via maternal milk and partly direct. Rat pups may gradually start to drink treated water from around PND 14, and on a mg-test-substance per kg-body-weight basis may actually be consuming a higher dose than adults during their second week of the lactation period [36]. It is needed to clarify the exposure levels of pups to PS80 produced adverse effects and clarify whether the adverse effects are attributable to the direct effects of PS80 on the developing nervous system or secondary effects via growth retardation.

In the present study, the toxicological effects were noted at 7.5% (16.783 ml/kg bw/day) and the NOAEL in this study was considered to be 1.0% (1.864 ml/kg bw/day). The value of the NOAEL is equivalent to 2013 mg/kg bw/day. It is estimated that daily intake of polysorbates from food is 12–111 mg/human in European and American countries [37]. The estimated human intake of polysorbates is equivalent to 0.20–1.85 mg/kg bw/day and is well below the NOAEL in this study.

Acknowledgment

This study was supported by grants from the Ministry of Health, Labour and Welfare, Japan.

References

[1] FDA (US Food and Drug Administration). Polysorbate 80. 21CFR172.840; 2006 [cited May 9, 2007]. Available from: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm.

[2] SIGMA. Product Information;TWEEN 80. 2007 [cited April 4, 2007]. Available from: http://www.sigmaaldrich.com/sigma/datasheet/s8686dat.pdf.

[3] Varma RK, Kaushal R, Junnarkar AY, et al. Polysorbate 80: a pharmacological study. Arzneimittelforschung 1985;35:804–8.

[4] Romaniuk A, Strzelczuk M, Wieczorek M. Is it advisable to use sorbitans in the investigation of the brain mechanisms? Acta Neurobiol Exp (Wars) 1989;49:249–54.
[5] Romaniuk A, Strzelczuk M, Wieczorek M. Further evidence against using sorbitans in the brain mechanisms investigation. Acta Neurobiol Exp (Wars) 1990;50:619–27.
[6] Brubaker CM, Taylor DH, Bull RJ. Effect of Tween 80 on exploratory behavior and locomotor activity in rats. Life Sci 1982;30:1965–71.
[7] OECD (Organization for Economic Co-operation and Development), OECD Guideline for the Testing of Chemicals, Draft Proposal for New Guideline 426, Developmental Neurotoxicity Study, August 18, 2005.
[8] MHLW (Ministry of Health, Labour and Welfare, Japan), Good Laboratory Practice Standards for Non-clinical Safety Studies on Drugs. MHLW ordinance No. 21, March 26, 1997.
[9] Low for Partial Amendments to the Law Concerning the Protection and Control of Animals Low No. 68, June 22, 2005.
[10] Snedecor G, Cochran W. Statistical methods. 7th ed. Iowa: Iowa State University Press; 1980.
[11] Dunnett C. A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc 1995;50:1096–121.
[12] Oser BL, Oser M. Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. III. Clinical and metabolic observations. J Nutr 1957;61:149–66.
[13] Artman NR. Safety of emulsifiers in fats and oils. J Am Oil Chem Soc 1975;52:49–52.
[14] CIR (Cosmetic Ingredient Review). Final report on the safety assessment of polysorbates 20, 21, 40, 60, 61, 65, 80, 81, and 85. J Am Coll Toxicol. 1984;3:1–43.
[15] KFDA (Korea Food and Drug Administration). Korea Food Additive Code, Specification and Standards #292. Polysorbate 80 [cited June 7, 2007]. Available from: http://fa.kfda.go.kr/standard/egongjeon_standard_list.jsp.
[16] Krantz Jr JC, Culver PJ, Carr CJ, Jones CM. Sugar alcohols-XXVIII. Toxicologic, pharmacodynamic and clinical observations on TWEEN 80. Bull Med Univ Sch MD 1951;36:48–56.
[17] Oser BL, Oser M. Nutritional studies on rats of diets containing high levels of partial ester emulsifiers. II. Reproduction and lactation. J Nutr 1956;60:489–505.
[18] NTP(National Toxicology Program). Developmental Toxicology of Polyoxyethylene Sorbitan Monooleate (CAS #9005-65-6) in Sprague–Dawley CD® Rats (NTP Study: TER91009); 1992 [cited March 16, 2007]. Available from: http://ntp.niehs.nih.gov/index.cfm?objectid=0730AFC6-AB6F-8A04-CFF2C1037A209CB8.
[19] Kavlock RJ, Short Jr RD, Chernoff N. Further evaluation of an in vivo teratology screen. Teratog Carcinog Mutagen 1987;7:7–16.
[20] Kameyama Y, Tanimura T, Yasuda M, et al. Spontaneous malformations in laboratory animals-photographic atlas and reference data. Cong Anom 1980;20:25–106.
[21] Morita H, Ariyuki F, Inomata N, et al. Spontaneous malformations in laboratory animals: frequency of external, internal and skeletal malformations in rats, rabbits and mice. Cong Anom 1987;27:147–206.
[22] Nakatsuka T, Hiromoto M, Ito M, Matsubara Y, Akaike M, Ariyuki F. Japan Pharmaceutical Manufacturers Association (JPMA) survey on background control data of developmental and reproductive toxicity studies in rats, rabbits and mice. Cong Anom 1997;37:47–138.
[23] Barnett Jr JF, Lewis D, Tappen A, Hoberman AM, Christian MS. Reproductive indices, fetal gross, visceral and skeletal alterations, sexual. Maturation, passive avoidance and water maze data, a comparison of results in CD(SD)IGS rats and CD(SD) rats. In: Matsuzawa T, Inoue H, editors. Biological reference data on CD(SD)IGS rats-2000, CD(SD)Igs study group. Yokohama: Charles River Japan, Inc.; 2000. p. 159–73.
[24] Crofton KM, Taylor DH, Bull RJ, Sivulka DJ, Lutkenhoff SD. Developmental delays in exploration and locomotor activity in male rats exposed to low level lead. Life Sci 1980;26:823–31.
[25] Culver B, Norton S. Juvenile hyperactivity in rats after acute exposure to carbon monoxide. Exp Neurol 1976;50:80–98.
[26] Evans HL, Bushnell PJ, Taylor JD, Monico A, Teal JJ, Pontecorvo MJ. A system for assessing toxicity of chemicals by continuous monitoring of homecage behaviors. Fundam Appl Toxicol 1986;6:721–32.
[27] Mondon CE, Dolkas CB, Sims C, Reaven GM. Spontaneous running activity in male rats: effect of age. J Appl Physiol 1985;58:1553–7.
[28] Ema M, Itami T, Kawasaki H. Behavioral effects of acute exposure to tributyltin chloride in rats. Neurotoxicol Teratol 1991;13:489–93.
[29] Ema M, Itami T, Kawasaki H. Changes of spontaneous motor activity of rats after acute exposure to tributyltin chloride. Drug Chem Toxicol 1991;14:161–71.
[30] Wirth-Dzieciolowska E, Lipska A, Wesierska M. Selection for body weight induces differences in exploratory behavior and learning in mice. Acta Neurobiol Exp (Wars) 2005;65:243–53.
[31] Lochry EA. Concurrent use of behavioral/functional testing in existing reproductive and developmental toxicity screens: practical considerations. J Am Coll Toxicol 1987;6:433–9.
[32] ICH (International Conference on Harmonisation), ICH Harmonised Tripartite Guideline: Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility S5(R2); 2005.
[33] Hass U. The need for developmental neurotoxicity studies in risk assessment for developmental toxicity. Reprod Toxicol 2006;22:148–56.
[34] Schardein JL, Macina OT. Human developmental toxicants: aspects of toxicology and chemistry. New York: CRC Press; 2006.
[35] Nelson BK. Evidence for behavioral teratogenicity in humans. J Appl Toxicol 1991;11:33–7.
[36] OECD (Organization for Economic Co-operation and Development). OECD Environment, Health and Safety Publications Series on Testing and Assessment, No. 43, Draft Guidance Document on Reproductive Toxicity Testing and Assessment, November 10, 2004.
[37] Food Safety Commission, Japan. Evaluation report: polysorbates; 2007.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food and Chemical Toxicology 43 (2005) 325–331
www.elsevier.com/locate/foodchemtox

Evaluation of developmental toxicity of 1-butanol given to rats in drinking water throughout pregnancy

M. Ema [a,*], H. Hara [b], M. Matsumoto [a], A. Hirose [a], E. Kamata [a]

[a] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Ina Research, Inc., 2148 Nishiminowa, Ina, Nagano 399-4501, Japan*

Received 21 September 2004; accepted 6 November 2004

Abstract

The objective of this study was to evaluate the developmental toxicity of 1-butanol in rats. Pregnant rats were given drinking water containing 1-butanol at 0.2%, 1.0% or 5.0% (316, 1454 or 5654 mg/kg/day) on days 0–20 of pregnancy. A significant decrease in maternal body weight gain accompanied by reduced food and water consumption was found at 5.0%. No significant increase in the incidence of pre- and postimplantation embryonic loss was observed in any groups treated with 1-butanol. Fetal weight was significantly lowered at 5.0%. Although a significant increase in the incidence of fetuses with skeletal variations and decreased degree of ossification was found at 5.0%, no increase in the incidence of fetuses with external, skeletal and internal abnormalities was detected in any groups treated with 1-butanol. The data demonstrate that 1-butanol is developmental toxic only at maternal toxic doses. No evidence for teratogenicity of 1-butanol was noted in rats. Based on the significant decreases in maternal body weight gain and fetal weight, it is concluded that the no observed adverse effect levels (NOAELs) of 1-butanol for both dams and fetuses are 1.0% (1454 mg/kg/day) in rats.

Keywords: 1-Butanol; Developmental toxicity; Teratogenicity; Fetal abnormality; Rat

1. Introduction

1-Butanol (CAS no. 71-36-3, *n*-butanol; *n*-butyl alcohol), a flammable colorless liquid with a rancid sweet odor, is widely used as an organic solvent and intermediate in the manufacture of other organic chemicals (IPCS/WHO, 1987). Exposure of the general population is mainly through its natural occurrence in food and beverages and its use as a flavoring agent (IPCS/WHO, 1987).

Several reports on the developmental toxicity of 1-butanol are available. Nelson et al. (1989a) reported the results of a developmental toxicity study in which SD rats were exposed to 1-butanol by inhalation for 7 hr/day on days 1–19 of pregnancy at 3500, 6000 and 8000 ppm (equivalent to estimated daily absorbed doses of 350, 600 and 800 mg/kg). They observed maternal deaths at 8000 ppm, decreases in maternal food consumption and fetal weight at 6000 and 8000 ppm, and an increased incidence of rudimentary cervical ribs at 8000 ppm, and concluded that 1-butanol was not a selective developmental toxicant in rats. Nelson et al. (1989b) conducted a behavioral teratology study in which female SD rats were given 1-butanol by inhalation at 3000 or 6000 ppm for 7 hr/day throughout pregnancy (the maternal exposure group); male rats were

Abbreviations: NOAEL, no observed adverse effect level

* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

0278-6915/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2004.11.003

similarly exposed for 6 weeks and mated to unexposed females (the paternal exposure group), and offspring were behaviorally and neurochemically examined. The data from all tests in their study were within the range of control data in other research conducted by their laboratory. Sitarek et al. (1994) reported a significant increase in the incidence of fetuses with abnormalities after administration of 1-butanol at 0.24–4.0% (300–5000 mg/kg/day) in drinking water during the pre-mating period for 8 weeks and throughout the mating and pregnant period. No maternal toxicity was found at any dose of 1-butanol. The no observed adverse effect level (NOAEL) was not derived from the results of their study, because significant increases in the incidence of fetuses with dilation of the subarachnoid space and dilation of the lateral ventricle and/or third ventricle of the brain were found even at the lowest dose (0.24%). They have concluded that 1-butanol is a developmental toxicant and produces anomalies in the skeleton and central nervous system.

The present study was conducted to determine whether or not morphological abnormalities could be produced in fetuses of rats given 1-butanol prenatally and designed to replicate the observations of the study by Sitarek et al. (1994).

2. Materials and methods

This study was performed in compliance with regulatory guidelines (MHW, 1997a) and accordance with the principles for Good Laboratory Practice (MHW, 1997b) and "Guidance for Animal Care and Use" of Ina Research, Inc.

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in reproductive and developmental toxicity studies and historical control data are available. Males at 10 weeks of age and females at 9 weeks of age were purchased from Tsukuba Breeding Center, Charles River Japan, Inc., (Yokohama, Japan). The rats were acclimated to the laboratory for 7 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a basal diet (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum and maintained in an air-conditioned room at 21–25 °C, with a relative humidity of 40–70%, a 12-h light/dark cycle, and ventilation with 16 air charges/hour. Virgin female rats were mated overnight with male rats. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats, weighing 217–273 g and 10–11 weeks of age, were distributed using a computerized randomization procedure (TOXstaff 21 system) into 4 groups of 20 rats each and housed individually.

2.2. Chemicals and dosing

1-Butanol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 1-butanol used in this study was 99.9% pure and a special grade reagent (Lot no. CER5688), and it was kept in a dark place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before and after the study. Rats were given 1-butanol in their drinking water at a concentration of 0 (control), 0.2%, 1.0% or 5.0% on day 0 through day 20 of pregnancy. The dosage levels were determined based on the results of our range-finding study in which administration of 1-butanol in the drinking water on days 0–20 of pregnancy caused decreases in maternal body weight gain and food and water consumption and tended to reduce in fetal weight at 4% and 7% in rats. 1-Butanol was dissolved in distilled water (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan). The control rats were given only water. The stability of formulations in a dark and cool place under airtight conditions has been confirmed for up to 3 days. During use, the formulations were maintained under such conditions for no more than 3 days and were 95.7–103.5% of the target concentration.

2.3. Observations

The maternal body weight and water consumption were recorded daily, and food consumption was recorded every 3 or 4 days. The pregnant rats were euthanized by exsanguinations under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the numbers of corpora lutea, implantation sites and live and dead fetuses and resorptions were counted. The live fetuses removed from the uterus were sexed, weighed, measured among their crown-rump length, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected and fixed in alcohol, stained with alizarin red S (Dawson, 1926) and examined for skeletal anomalies. The remaining live fetuses in each litter were fixed in Bouin's solution. Their heads were subjected to a free-hand razor-blade sectioning (Wilson, 1973) and the thoracic areas were subjected to microdissecting (Nishimura, 1974) to reveal internal abnormalities. The placental weight was also measured.

2.4. Data analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. The initial body

weight, body weight gain and food and water consumption of pregnant rats, numbers of corpora lutea, implantations and live fetuses per litter, fetal weight and crown-rump length and placental weight were analyzed with Bartlett's test (Snedecor and Cochran, 1980) for homogeneity of variance at the 5% level of significance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test (Dunnett, 1955) to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the mean rank of the 1-butanol-treated groups was compared with that of the control group with the Dunnett type test. The Dunnett type test was used for the incidences of pre- and postimplantation embryonic loss and fetal anomalies and sex ratio of fetuses to compare the mean rank of groups treated with 1-butanol and that of the control group. The incidence of dams with anomalous fetuses was analyzed by Chi-square test or Fisher's exact test. The significance of differences from the control group was estimated at probability levels of 1% and 5%.

3. Results

Table 1 shows the maternal findings in rats given 1-butanol during pregnancy. No death was found in female rats of any group. All females in all groups became pregnant. The body weight gains on days 0–7 of pregnancy were significantly reduced at 5.0%. The body weight gain during the whole period of pregnancy was also significantly decreased at 5.0%. No significant decrease in the body weight gain was noted at 0.2 or 1.0, except for a transient decrease on days 0–2 of pregnancy at 1.0%. The food consumption on days 0–7, days 7–14, days 14–20 and days 0–20 of pregnancy was significantly lower in the 1.0% and 5.0% groups than the control group. The water consumption on days 0–7 at 1.0 and 5.0% and on days 7–14, days 14–20 and days 0–20 at 5.0% was significantly decreased. The mean daily intakes of 1-butanol were 316 mg/kg for the 0.2% group, 1454 mg/kg for the 1.0% group and 5654 mg/kg for the 5.0% group.

Reproductive findings in rats given 1-butanol during pregnancy are presented in Table 2. No litters totally resorbed were found in any group. No effects of the administration of 1-butanol were observed on the numbers of corpora lutea, implantations, pre- or postimplantation loss, resorptions or dead or live fetuses or sex ratio of live fetuses. The body weights of male and female fetuses were significantly lower in the 5.0% group than in the control group. There was no significant difference in the crown-rump length of male and female fetuses or placental weight between the control and groups treated with 1-butanol.

A summary of morphological findings in live fetuses of rats given 1-butanol during pregnancy is shown in Table 3. One fetus with spina bifida in the control group and one fetus with thread-like tail and anal atresia in the 0.2% group were observed. Skeletal examination

Table 1
Maternal findings in rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
No. of rats	20	20	20	20
No. of pregnant rats	20	20	20	20
No. of dead rats	0	0	0	0
Initial body weight	245 ± 14	247 ± 13	245 ± 11	244 ± 12
Body weight gain during pregnancy (g)[a]				
Days 0–7	44 ± 7	45 ± 7	40 ± 6	20 ± 28**
Days 7–14	40 ± 6	41 ± 5	41 ± 7	42 ± 10
Days 14–20	78 ± 14	82 ± 8	84 ± 7	75 ± 11
Days 0–20	162 ± 19	168 ± 16	165 ± 15	146 ± 16**
Food consumption during pregnancy (g)[a]				
Days 0–7	179 ± 12	180 ± 16	164 ± 12*	138 ± 21**
Days 7–14	193 ± 14	194 ± 17	177 ± 14**	160 ± 11**
Days 14–20	176 ± 14	175 ± 15	161 ± 12**	143 ± 11**
Days 0–20	548 ± 38	548 ± 46	503 ± 34**	441 ± 34**
Water consumption during pregnancy (ml)[a]				
Days 0–7	284 ± 28	305 ± 37	258 ± 29*	175 ± 34**
Days 7–14	318 ± 35	337 ± 48	299 ± 40	239 ± 80**
Days 14–20	328 ± 47	342 ± 47	334 ± 46	256 ± 85**
Days 0–20	930 ± 105	983 ± 126	890 ± 106	669 ± 182**
Mean daily intakes of 1-butanol (mg/kg)[a]	0	316 ± 30	1454 ± 186	5654 ± 1402

*,** Significantly different from the control, *$P < 0.05$ and **$P < 0.01$.
[a] Values are given as the mean ± SD.

Table 2
Reproductive findings in rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
No. of litters	20	20	20	20
No. of litters totally resorbed	0	0	0	0
No. of corpora lutea per litter[a]	16.4 ± 3.6	16.7 ± 3.0[d]	16.1 ± 2.1	16.3 ± 2.6
No. of implantations per litter[a]	14.3 ± 2.8	15.1 ± 1.7	15.2 ± 1.2	14.7 ± 2.5
% Preimplantation loss per litter[b]	9.0	9.0[d]	4.4	9.2
% Postimplantation loss per litter[c]	6.0	5.4	3.7	8.0
No. of live fetuses per litter[a]	13.4 ± 2.6	14.3 ± 1.4	14.7 ± 1.5	13.5 ± 2.5
Sex ratio of live fetuses (male/female)	128/139	145/140	149/144	131/139
Body weight of live fetuses (g)[a]				
Male	4.18 ± 0.27	4.00 ± 0.24	4.04 ± 0.25	3.83 ± 0.18**
Female	3.97 ± 0.25	3.86 ± 0.20	3.83 ± 0.16	3.59 ± 0.17**
Fetal crown-rump length (mm)[a]				
Male	40.5 ± 1.2	40.3 ± 1.4	40.2 ± 1.2	39.7 ± 1.3
Female	39.4 ± 1.2	39.4 ± 1.2	39.3 ± 1.1	38.5 ± 1.4
Placental weight (g)				
Male	0.50 ± 0.05	0.49 ± 0.05	0.48 ± 0.06	0.50 ± 0.06
Female	0.49 ± 0.05	0.48 ± 0.05	0.47 ± 0.05	0.49 ± 0.06

** Significantly different from the control, $P < 0.01$.
[a] Values are given as the mean ± SD.
[b] (No. of preimplantation embryonic loss/no. of corpora lutea) × 100.
[c] (No. of resorptions and dead fetuses/no. implantations) × 100.
[d] Value was obtained from 19 pregnant rats.

revealed one fetus with supernumerary thoracic vertebral bodies and malpositioned thoracic vertebrae at 1.0%. Although the total number of fetuses with skeletal variations was significantly increased at 5.0%, the number of fetuses with individual skeletal variations was not significantly increased, except for fetuses with short supernumerary ribs at 5.0%. A significantly lower number of forepaw proximal phalanges was observed at 5.0%. Membranous ventricular septum defect occurred in one fetus of the control and 0.2% groups and 3 fetuses in 3 dams of the 5.0% group. One fetus with a double aorta in the control group and one fetus with a left umbilical artery in the control and 2.0% groups were observed. Thymic remnants in the neck were found in 4–11 fetuses of the control and groups treated with 1-butanol. However, there was no significant difference in the incidence of fetuses with internal abnormalities between the control and groups treated with 1-butanol.

4. Discussion

The present study was conducted to determine the developmental toxicity of 1-butanol and designed to replicate the observations of the study by Sitarek et al. (1994). The data showed that prenatal administration of 1-butanol did not produce morphological anomalies in fetuses of rats. Thus, we have been unable to confirm the results of Sitarek's study in which prenatal exposure to 1-butanol produced fetal anomalies.

The doses of 1-butanol used in the present study expected to induce maternal and/or developmental toxicity, such as a decrease in maternal body weight gain and fetal weight, were given to pregnant rats during the whole period of pregnancy to characterize the effects of 1-butanol on embryonic/fetal development. Maternal toxicity, a significant decrease in body weight gain, was found at 5.0%. Maternal food and water consumptions were also reduced in this dose group. Although the only significant decrease in maternal body weight gain was observed on days 0–2 of pregnancy at 1.0%, this decrease was occasional and discontinuous and seems unlikely to be of toxicological significance. In this dose group, decreases in the maternal food consumption during the whole period of pregnancy and water consumption during the early period of pregnancy, which were unaccompanied by the continuous changes in body weight gain, were observed. No significant changes in maternal parameters were noted in the 0.2% group. These findings in maternal rats indicate that 1-butanol exerts maternal toxicity at 5.0% (equivalent to 5654 mg/kg/day) when administered during the entire period of pregnancy in rats.

No significant increase in the incidence of postimplantation loss was found at any dose of 1-butanol, and significantly decreased weights of male and female fetuses were found at 5.0%. No significant adverse effects on reproductive parameters were detected at 0.2% and 1.0%. These findings indicate that 1-butanol is not toxic to embryonic/fetal survival up to 5.0% or fetal growth up to 1.0% when administered during the whole period of pregnancy.

As for morphological examinations in the fetuses of exposed mothers, a few fetuses with external, skeletal

Table 3
Morphological examinations in fetuses of rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
External examination				
Total no. of fetuses (litters) examined	267 (20)	285 (20)	293 (20)	270 (20)
Total no. of fetuses (litters) with abnormalities	1 (1)	1 (1)	0	0
Spina bifida	1 (1)	0	0	0
Thread-like tail and anal atresia	0	1 (1)	0	0
Skeletal examination				
Total no. of fetuses (litters) examined	139 (20)	147 (20)	152 (20)	140 (20)
Total no. of fetuses (litters) with abnormalities	0	0	1 (1)	0
Supernumerary of thoracic vertebral bodies and malpositioned thoracic vertebrae	0	0	1 (1)	0
Total no. of fetuses (litters) with variations	28 (11)	23 (12)	52 (17)	69 (20)**
Bipartite ossification of thoracic centra	1 (1)	1 (1)	1 (1)	7 (5)
Dumbbell ossification of thoracic centra	0	1 (1)	2 (2)	3 (3)
Bipartite ossification of lumbar centra	0	0	0	2 (2)
Supernumerary lumbar vertebrae	4 (1)	1 (1)	5 (3)	5 (2)
Lumbarization	0	0	1 (1)	1 (1)
Bipartite ossification of sternebrae	1 (1)	1 (1)	1 (1)	1 (1)
Misaligned sternebrae	0	0	0	1 (1)
Cervical ribs	2 (2)	3 (3)	3 (3)	7 (5)
Full supernumerary ribs	5 (2)	1 (1)	10 (5)	9 (5)
Short supernumerary ribs	20 (10)	18 (9)	43 (16)	55 (19)**
Wavy ribs	0	0	0	1 (1)
Degree of ossification[a]				
No. of sacral and caudal vertebrae	8.4 ± 0.5	8.4 ± 0.4	8.3 ± 0.5	8.1 ± 0.3
No. of sternebrae	5.9 ± 0.2	5.8 ± 0.2	5.8 ± 0.2	5.8 ± 0.2
No. of forepaw proximal phalanges	1.6 ± 1.3	1.6 ± 0.9	1.2 ± 1.1	0.3 ± 0.4**
Internal examination				
Total no. of fetuses (litters) examined	128 (20)	138 (20)	141 (20)	130 (20)
Total no. of fetuses (litters) with abnormalities	7 (6)	9 (6)	11 (8)	14 (9)
Membranous ventricular septum defect	1 (1)	1 (1)	0	3 (3)
Double aorta	1(1)	0	0	0
Left umbilical artery	1 (1)	0	1 (1)	0
Thymic remnant in neck	4 (4)	8 (5)	10 (8)	11 (8)

** Significantly different from the control, $P < 0.01$.
[a] Values are given as the mean ± SD.

and/or internal abnormalities were found in all groups. The abnormalities observed in the present study are not thought to be due to the administration of 1-butanol, because they have occurred at a very low incidence and are of types that occur sporadically among control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). Several types of skeletal variations were also found in the control and groups treated with 1-butanol. These skeletal variations are frequently observed in fetuses of rats at term (Kimmel and Wilson, 1973; Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). In the 5.0% group, a significant increase in the incidence of fetuses with skeletal variations and fetuses with short supernumerary ribs, but not full supernumerary ribs, and a significant decrease in the degree of ossification were accompanied by a significant decrease in the fetal weight. These findings show a correlation between these morphological alterations and growth retardation in fetuses. Although a skeletal variation, i.e., full supernumerary ribs, is a warning sign of possible teratogenicity, short supernumerary ribs, sternebral variations, and bilobed centra of the vertebral column are normal variations (Kimmel and Wilson, 1973). Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and this might result from a delay in growth or morphogenesis that has otherwise followed a normal pattern of development. Consideration of these findings together suggests that the morphological changes in fetuses observed in the present study do not indicate a teratogenic response and that 1-butanol possesses no teratogenic potential in rats.

In Sitarek's study (1994), significant increases in the incidences of wavy ribs at 300 mg/kg/day, dilation of the subarachnoid space and dilation of the lateral ventricle and/or third ventricle of the brain at 300 mg/kg/day and higher, dilation of the renal pelvis and external hydrocephaly at 1000 mg/kg/day, internal hydrocephaly at 1000 mg/kg/day and higher, and supernumerary ribs and delayed ossification at 5000 mg/kg/day were found. A significant decrease in fetal crown-rump length was

also observed at 5000 mg/kg/day. Based on these findings, Sitarek et al. (1994) concluded that 1-butanol had adverse effects on the morphological development of fetuses in rats. However, we did not confirm their findings. We have demonstrated here that prenatal 1-butanol has no adverse effect on the morphological development of rat offspring. There are some differences between Sitarek's study and the present study in experimental conditions, such as duration of administration and rat strain used in the experiments. Sitarek et al. (1994) administered 1-butanol to female rats for 8 weeks before mating and throughout the mating and pregnancy period and found fetal anomalies, such as hydrocephaly and dilation of the cerebral ventricles and the renal pelvis. On the other hand, we gave 1-butanol to female rats during the whole period of pregnancy and did not detect fetuses with these anomalies. Administration during the premating and mating period is thought to be excluded from the susceptible period for induction of morphological anomalies such as hydrocephaly/dilation of the cerebral ventricles and dilation of the renal pelvis, because rat fetuses are susceptible to induction of these anomalies during mid and late pregnancy (Wood and Hoar, 1972; Kameyama, 1985). The strain difference of rats used in the experiments may explain the discrepancy in the findings regarding fetal anomalies between the studies. In Sitarek's study (1994), ImP: DAK rats obtained from their own breeding colony were used. No detailed information on this strain of rats was available (Sitarek et al., 1994). In their study, dilation of the lateral ventricle and/or third ventricle of the brain was observed in 2% of fetuses (one of the 12 litters) in the control group. In their another study using Imp: DAK rats, extension of the lateral ventricle and/or third ventricle of the brain was observed in 11.7% of fetuses (8 of the 17 litters) in the control group (Sitarek et al., 1996). However, these anomalies were not found in the control group of their studies using Wistar rats (Baranski et al., 1982), Imp: Lodz rats (Sitarek, 1999, 2001) and Imp: WIST rats (Sitarek and Sapota, 2003). The incidences of dilation of the cerebral ventricles in Imp: DAK rats are thought to be higher than those in the background control data of other strains of rats. The fetal incidence of hydrocephaly/dilation of cerebral ventricles in the control rats of reproductive studies conducted between 1986 and 1993 in 63 research institutes is reported to be 0–0.09% and 0–0.26%, respectively (Nakatsuka et al., 1997). In Crj: CD (SD) IGS rats which were used in the present study, the incidence of dilation of the lateral ventricles of the brain in 19 studies conducted during 1998–2000 is reported to be 0–0.06% in fetuses and 0–0.44% in litters (Barnett et al., 2000). Thus, hydrocephaly/dilation of the cerebral ventricle is not commonly observed in fetuses of common strains of rats.

The difference in terminology used for classification of structural anomalies in fetuses may also explain the discrepancy in the findings regarding fetal anomalies between the studies. Sitarek et al. (1996) stated that minor abnormalities, such as enlarged lateral ventricle and/or third ventricle, are quite frequent in rat fetuses and without having the dose-dependent relationship should not be taken alone as evidence of tested chemical fetotoxicity. However, the Fourth Berlin Workshop on Terminology in Developmental Toxicity noted that changes affecting brain ventricles are more likely to be classified as malformations and classification should be based on the historical control incidences, the nature of the organ affected and the severity (Solecki et al., 2003). In Sitarek's study (1994), dilation of the subarachnoid space was observed in fetuses of rats given 1-butanol at 300 mg/kg/day and higher. This anomaly was also found in fetuses in Imp: DAK rats given *N*-cyclohexyl-2-benzothiazolesulfenamide (Sitarek et al., 1996) and Imp: Lodz rats given *N*-methylmorpholine (Sitarek, 1999). No information on the definition of this anomaly was available in their reports. We are unaware of this anomaly in other literature (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Horimoto et al., 1998; Barnett et al., 2000; Solecki et al., 2003).

In conclusion, the administration of 1-butanol to pregnant rats throughout pregnancy had adverse effects on maternal rats and embryonic/fetal growth but had no adverse effects on fetal morphological development even at a maternally toxic dose. The data indicate that 1-butanol induces developmental toxicity only at maternally toxic doses in rats. Based on the significant decreases in maternal body weight gain and fetal weight at 5.0%, it is concluded that the NOAELs of 1-butanol for both dams and fetuses are 1454 mg/kg/day (1.0% in drinking water) in rats.

Acknowledgment

This study was performed in 2003 through 2004 at the Ina Research, Inc. (Ina, Japan) and supported by the Ministry of Health, Labour and Welfare, Japan.

References

Baranski, B., Stetkiewicz, I., Trzcinka-Ochocka, M., Sitarek, K., Szymczak, W., 1982. Teratogenicity, fetal toxicity and tissue concentration of cadmium administered to female rats during organogenesis. Journal of Applied Toxicology 2, 255–259.

Barnett Jr., J.F., Lewis, D., Tappen, A., Hoberman, A.M., Christian, M.S., 2000. Reproductive indices, fetal gross, visceral and skeletal alterations, sexual maturation, passive avoidance and water maze data, a comparison of results in CD(SD)IGS rats and CD(SD) rats. In: Matsuzawa, T., Inoue, H. (Eds.), Biological Reference Data on CD (SD)IGS Rats-2000. CD(SD)IGS Study Group, c/o Charles River Japan Inc., Yokohama, Japan.

Chahoud, I., Buschmann, J., Clark, R., Druga, A., Falke, H., Faqi, A., Hansen, E., Heinrich-Hirsch, B., Helleig, J., Lingk, W., Parkinson,

M., Paumgartten, F.J.R., Pefil, R., Platzek, T., Scialli, A.R., Seed, J., Stahlmann, R., Ulbrich, B., Wu, X., Yasuda, M., Younes, M., Solecki, R., 1999. Classification terms in developmental toxicology: need for harmonization. Report of the second workshop on the terminology in developmental toxicology Berlin, 27–28 August 1998. Reproductive Toxicology 13, 77–82.

Dawson, A.B., 1926. A note on the staining of the skeleton of cleared specimens with arizarin red-S. Stain Technology 1, 123–124.

Dunnett, C.W., 1955. A multiple comparison procedure for comparing several treatments with control. Journal of American Statistical Association 50, 1096–1121.

Horimoto, M., Ariyuki, F., Daidohji, S., Fujii, T., Fukunishi, K., Hanada, S., Ikegami, S., Ishii, H., Inoue, T., Iwase, T., Matsuura, M., Matsuzawa, T., Nishi, N., Ohkubo, Y., Sanbuissho, A., Sekiya, K., Tani, M., Taniguchi, H., Yokomoto, Y., Yoshida, J., Takahashi, M., Yasuda, M., 1998. Terminology of developmental abnormalities in common laboratory mammals (Japanese version 1). Congenital Anomalies 38, 153–237 (Japanese).

IPCS/WHO (International Programme on Chemical Safety/World Health Organization), 1987. Environmental Health Criteria 65. Butanols: Four Isomers: 1-Butanol, 2-Butanol, *tert*-Butanol, Isobutanol. WHO, Geneva.

Kameyama, Y., 1985. Comparative developmental pathology of the central nervous system. In: Marois, M. (Ed.), Prevention of Physical and Mental Congenital Defects. Part A: The Scope of the Problem. Alan R. Liss, New York.

Kameyama, Y., Tanimura, T., Yasuda, M. (Eds.), 1980. Spontaneous malformations in laboratory animals-photographic atlas and reference data. Congenital Anomalies 20, 25–106 (Japanese).

Kimmel, C.A., Wilson, G.J., 1973. Skeletal deviations in rats: Malformations or variations? Teratology 8, 309–316.

MHW, Japan (Ministry of Health and Welfare, Japan), 1997a. Guidelines for Toxicity Studies of Drugs.

MHW, Japan (Ministry of Health and Welfare, Japan), 1997b. The GLP Standards for Non-clinical Safety Studies on Drugs, MHW Ordinance no. 21.

Morita, H., Ariyuki, F., Inomata, N., Nishimura, K., Hasegawa, Y., Miyamoto, M., Watanabe, T., 1987. Spontaneous malformations in laboratory animals: frequency of external, internal and skeletal malformations in rats, rabbits and mice. Congenital Anomalies 27, 147–206.

Nakatsuka, T., Horimoto, M., Ito, M., Matsubara, Y., Akaike, M., Ariyuki, F., 1997. Japan Pharmaceutical Manufacturers Association (JPMA) survey on background control data of developmental and reproductive toxicity studies in rats, rabbits and mice. Congenital Anomalies 37, 47–138.

Nelson, B.K., Brightwell, W.S., Khan, A., Burg, J.R., Goad, P.T., 1989a. Lack of selective developmental toxicity of three butanol isomers administered by inhalation to rats. Fundamental and Applied Toxicology 12, 469–479.

Nelson, B.K., Brightwell, W.S., Robertson, S.K., Khan, A., Krieg Jr., E.F., Massari, V.J., Burg, 1989b. Behavioral teratology investigation of 1-butanol in rats. Neurotoxicology and Teratology 11, 313–315.

Nishimura, K., 1974. A microdissection method for detecting thoracic visceral malformations in mouse and rat fetuses. Congenital Amomalies 14, 23–40 (Japanese).

Sitarek, K., 1999. Maternal and fetal toxicity of *N*-methylmorpholine by oral administration in rats. Teratogenesis, Carcinogenesis, and Mutagenesis 19, 369–376.

Sitarek, K., 2001. Embryolethal and teratogenic effects of carbendazim in rats. Teratogenesis, Carcinogenesis, and Mutagenesis 21, 335–340.

Sitarek, K., Berlinska, B., Baranski, B., 1994. Assessment of the effect of *n*-butanol given to female rats in drinking water on fertility and prenatal development of their offspring. International Journal of Occupational Medicine and Environmental Health 7, 365–370.

Sitarek, K., Berlinska, B., Baranski, B., 1996. Effect of oral Sulfenamide TS administration on prenatal development in rats. Teratogenesis Carcinogenesis and Mutagenesis 16, 1–6.

Sitarek, K., Sapota, A., 2003. Maternal-fetal distribution and prenatal toxicity of 2,2,4-trimethyl-1,2-dihydroquinoline in the rat. Birth Defects Research, Part B 68, 375–382.

Snedecor, G.W., Cochran, W.G., 1980. Statistical Methods, seventh ed. Iowa State University Press.

Solecki, R., Bergmann, B., Bürgin, H., Buschmann, J., Clark, R., Druga, A., Van Duijnhoven, E.A.J., Duverger, M., Edwards, J., Freudenberger, H., Guittin, P., Hakaite, P., Heinrich-Hirsch, B., Hellwig, J., Hofmann, T., Hübel, U., Khalil, S., Klaus, A., Kudicke, S., Lingk W., Meredith, T., Moxon, M., Müller, S., Paul, M., Paumgartten, F., Röhrdanz, E., Pfeil, R., Rauch-Ernst, M., Seed, J., Spezia, F., Vickers, C., Woelffel, B., Chahoud, I., 2003. Harmonization of rat fetal external and visceral terminology and classification: Report of the Fourth Workshop on the Terminology in Developmental Toxicology, Berlin, 18–20 April 2002. Reproductive Toxicology, 17, 625–637.

Wilson, J.G., 1973. Methods for administering agents and detecting malformations in experimental animals. In: Wilson, J.G., Warkany, J. (Eds.), Teratology: Principles and Techniques. The University of Chicago Press, Chicago, pp. 262–277.

Wood, D.C., Hoar, R.M., 1972. Apparently hydronephrosis as a normal aspect of renal development in late gestation of rats: the effect of methyl salicylate. Teratology 6, 191–196.

Metals, Fertility, and Reproductive Toxicity

Edited by
Mari S. Golub

Taylor & Francis
Taylor & Francis Group
Boca Raton London New York

A CRC title, part of the Taylor & Francis imprint, a member of the Taylor & Francis Group, the academic division of T&F Informa plc.

Published in 2006 by
CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number-10: 0-415-70040-X (Hardcover)
International Standard Book Number-13: 978-0-415-70040-5 (Hardcover)
Library of Congress Card Number 2005049128

Library of Congress Cataloging-in-Publication Data

Metals, fertility, and reproductive toxicity / edited by Mari Golub.
p. cm.
Includes bibliographical references.
ISBN 0-415-70040-X (alk. paper)
1. Metals--Toxicology. 2. Reproductive toxicology. I. Golub, Mari S.

RA1231.M52M53 2005
615.9'253--dc22 2005049128

informa
Taylor & Francis Group
is the Academic Division of Informa plc.

Visit the Taylor & Francis Web site at
http://www.taylorandfrancis.com

and the CRC Press Web site at
http://www.crcpress.com

chapter 3

Reproductive and Developmental Toxicity of Organotin Compounds

Makoto Ema and Akihiko Hirose
Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan

Contents

Introduction

Organotin compounds are chemicals widely used in agriculture and industry (Piver 1973, World Health Organization 1980). Tetrasubstituted organotin compounds are mainly used as intermediates in the preparation of other organotin compounds. Trisubstituted organotin compounds have biocidal properties and are used in agriculture as fungicides and acaricides, as rodent repellents, and mulluscicides, and are widely used as antifoulants in ship paints and underwater coatings. Especially, triphenyltins (TPTs) and tributyltins (TBTs) have been used extensively in antifouling products such as algaecides and mulluscicides. Disubstituted organotin compounds are commercially the most important derivatives, and are mainly used in the plastics industry, particularly as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers. Monosubstituted organotin compounds are used as stabilizers in PVC films. Widespread use of organotin compounds has caused increasing amounts to be released into the environment. The most important nonpesticidal route of entry for organotin compounds into the environment is through leaching of organotin-stabilized PVC in water (Quevauviller et al. 1991), and the use in antifouling agents, resulting in the introduction of organotin into the aquatic environment (Maguire 1991). Data are available regarding the detection of butyltin and phenyltin compounds in aquatic marine organisms (Sasaki et al. 1988, Fent and Hunn 1991, Lau 1991) and marine products (Suzuki et al. 1992, Belfroid et al. 2000, Tsuda et al. 1995, Ueno et al. 1999, Toyoda et al. 2000). Food chain bioamplification of butyltin in oysters (Waldock and Thain 1983), mud crabs (Evans and Laughlin 1984), marine mussels (Laughlin et al. 1986), Chinook salmon (Short and Thrower 1986), and dolphin, tuna, and shark (Kannan et al. 1996), and of phenyltin in carp (Tsuda et al. 1987) and horseshoe crab (Kannan et al. 1995) has been reported. These indicate that organotin compounds accumulate in the food chain and are bioconcentrated, and that humans can be exposed to organotin compounds via seafood. The World Health Organization (WHO) reported in 1980 that the estimated mean total daily intake of tin by humans ranged from 200 μg to 17 mg. Recently, Tsuda et al. (1995) reported that the daily intakes in Shiga prefecture in Japan were 0.7 to 5.4 μg in 1991 and 0.7 to 1.3 μg in 1992 for TPT and 4.7 to 6.9 μg in 1991 and 2.2 to 6.7 μg in 1992 for TBT. Toyoda et al. (2000) also showed that the daily intakes in Japanese consumers, based on analysis with the 1998 total diet samples, were 0.09 μg for TPT, 0 μg for diphenyltin (DPT), 1.7 μg for TBT, and 0.45 μg for dibutyltin (DBT). These values are lower than the acceptable daily intake for TPT according to the JMPR (Joint Meetings of the FAO [Food and Agriculture Organization] and World Health Organization

Panel of Experts on Pesticides Residues), 25 µg (World Health Organization 1992), and the guidance value for oral exposure to tributyltin oxide (TBTO), 18 µg (International Programme on Chemical Safety 1999a). Thus, the levels of organotin compounds in seafood are not considered to be sufficiently high to affect human health (Tsuda et al. 1995, Ueno et al. 1999). However, Belfroid et al. (2000) noted that more research on residual TBT levels in seafood is needed before a definitive conclusion on possible health risks can be drawn.

In recent years, adverse effects of environmental chemicals on the reproductive success of wildlife populations have been reported (Colborn et al. 1993). These phenomena may result from interference with the endocrine system. Disturbances of hormonal regulation during pre- and postnatal development may produce deleterious effects on reproduction and development. TPT and TBT are suspected to be endocrine disruptors (Japan Environment Agency 1998). TBT and TPT are known to have strong effects on the development of imposex (imposition of male sex characteristics on females) in the rock shell (Horiguchi et al. 1996, 1997a), and this condition may bring about reproductive failure and a consequent population decline.

Although the toxicity of organotins has been extensively reviewed (World Health Organization 1980, Snoeij et al. 1987, Winship 1988, Boyer 1989, International Programme on Chemical Safety 1999a, b), the reproductive and developmental toxicity of these compounds is not well understood. In this chapter, we summarize the findings of the studies on reproductive and developmental effects of organotin compounds.

Effects on Aquatic Organisms

Imposex on Gastropods

TBT causes reproductive toxic effects in marine gastropods, which were represented by some masculinizing effects including *imposex* or *pseudohermaphodi(ti)sm*. The imposition of male sex organs (a penis and vas deferens) on female mud snails (*Nassarius obsoletus*) was found in near harbors, and the degree of penis development and frequency of imposex were positively correlated to the seawater TBT concentration (Smith 1981a, b). Imposex has been induced experimentally by treatment with 4.5 to 5.5 µg/L of TBT compounds for 60 days. In field studies in southeastern England, imposex has been reported in declining populations of the common dogwhelk (Bryan et al. 1986, 1987, 1989, Gibbs and Bryan 1986, Davies et al. 1987, Gibbs et al. 1987).

Imposex has not just occurred at a regional level, but worldwide on a global scale. Imposex in dogwhelk was not only reported in England, but in Scotland, the Netherlands, and the coastline of the North Sea. Imposex in other whelk species occurred in Canada, West Africa, New Zealand, Australia, Malaysia, Singapole, Indonesia, and Japan (Fent 1996, Horiguchi et al. 1996). Imposex among prosobranchs is known to occur in around 70 species of 50 genera, although some species are less susceptible to TBT compounds (Fioroni et al. 1991, Fent 1996).

TPT also induced imposex in *Thais clavigera* at the same potency as TBT (Horiguchi et al. 1997a). Although, in *Nucwlla lapillus*, TPT did not induce imposex, tripropyltin (TPrT) had a small effect on the development of imposex (Bryan et al. 1988). DBT and monobutyltin (MBT) did not induce imposex in the gastropod species examined. Three trisubstitution compounds (TBT, TPT, TPrT) and monophenyltin (MPT) easily induced imposex in some species, among the eight organotins, i.e., MBT, DBT, TBT, tetrabutyltin (TeBT), MPT, DPT, TPT, and TPrT. (Bryan et al. 1988, Hawkins and Hutchinson 1990, Horiguchi et al. 1997b).

The early studies in the 1980s reached some common conclusions, which are described below (Eisler 2000). Imposex correlated with the body burden of tributyl- and dibutyltin, but not with the tissue concentration of arsenic, cadmium, copper, lead, silver, or zinc. Forty-one percent of females had male characteristics, when the body burden reached to 1.65 mg Sn/kg of dry soft parts, by exposing with 0.02 μg Sn/L for 120 days. Imposex in immature females is caused above the concentration of around 1 ng/L (Sn) in seawater. At higher concentrations of TBT, the oviduct had been blocked, resulting in sterilization. Declining dogwhelk populations could be caused by aborting capsules, sterility, and premature death, which were characterized by a moderate to high degree of imposex, fewer female functions, fewer juveniles, and scarcity of laid egg capsules.

There is also a great variety of gradations of imposex in different species. The intensity is characterized by a classification system, which distinguishes six stages with a few different types, mainly based on a Vas Deferens Sequence (VDS) index (Oehlmann et al. 1991). Imposex development occurred in three variations: (1) a small penis without penis duct, (2) a short distal vas deferens section, or (3) a short proximal vas deferens section (stage 1). At stages 2 and 3 the male sex characteristics of each type are developed continuously. Stage 4 is characterized by a penis with penis duct and a complete vas deferens, and represents the last stage of fertility. The reproductive failure or sterility is induced in later stages. At stage 5 the vagina is replaced with a small prostate gland, the vagina opening is blocked by vas deferens tissue, or the incompletion of the pallial oviduct closure occurs. Abortive egg capsules fill the lumen and vestibulum of the capsule gland and evoke an intense swelling of the gland at stage 6 (Bettin et al. 1996). High TBT exposure in the early stages of life induced gametogenesis or sex changes characterized by a suppression of oogenesis and commencement of spermatogenesis in females (Gibbs et al. 1988, Fioroni et al. 1991, Oehlmann et al. 1991, 1996, Horiguchi et al. 2002). It was thought that the initial phases of imposex corresponding to VDS stages 1 and 2 may be reversible; however, advanced phases of imposex and sterilization with gross morphological changes corresponding to VDS stage 5 and 6 would be irreversible (Fent 1996).

Although many morphological aspects of pseudohermaphodi(ti)sm caused by TBT have been investigated, the biochemical mechanism has been indistinct. It is known that a neurotropic hormone called the penis morphogenic factor (PMF) develops male normal differentiation in mollusks (Féral

and LeGall, 1983). Co-localization of TBT with PMF in ganglia suggested that PMF release through TBT's neurotropic action induced masculinization in females (Bryan et al. 1989). Other studies indicated increased testosterone levels detected in female dogwhelk exposed to TBT, and that testosterone injection without TBT induced penis development in females (Spooner et al. 1991, Stroben et al. 1991). The later studies suggested that TBT disturbed the P-450–dependent aromatization of androgens to estrogen, and a nonsteroidal specific aromatase inhibitor–induced imposex similar to TBT (Bettin et al. 1996). However, the PMF has not been well characterized, and the role of vertebrate sex steroids is not known in gastropods to date. A recent study proposed that the combination of changes in the neuropeptide (APGWamide), which is considered to be a PMF in mud snails, and steroid hormones would lead to imposex induction at extremely low doses of TBT (Oberdörster and McClellan-Green 2002).

Effects on Fish

TBT or TPT exposure in early life stages induces altered embryonic development, and delayed or inhibited hatching in fish. Exposure of TBT or TBT to minnow eggs and larvae at concentrations of 0.2 to 18 µg/L in the water in which the fish lived induced dose-dependent morphological effects on larvae. Marked body axis deformations were observed at more than about 4 µg/L exposure, and incomplete hatching occurred at similar concentrations in 10 to 30% of larvae. At 15.9 µg/L of TPT exposure, hatching was delayed and the hatching rate was reduced significantly (Fent and Meier 1992, 1994). Developmental defects, such as skeletal abnormality and retarded yolk sac resorption, occurred in zebrafish larvae at more than 25 µg/L of triphenyltin acetate (TPTA) exposure, and hatching delay was found at more than 0.5 µg/L (Strmac and Braunbeck 1999). These developmental effects in fish were caused not only by organotin compounds, but also by a variety of contaminants (i.e., heavy metals, chlorinated hydrocarbons, altered pH), suggesting that such alteration would be classified as a nonspecific reaction to organic toxicants (Fent 1996, Strmac and Braunbeck 1999).

Some reproductive effects (i.e., reduced fecundity and sperm counts) in fish were reported. Reproductive success of three-spine stickleback with TBT exposure were examined over a 7-month period; no effects were detected in relation to fecundity, number of hatched fry, or frequency of malformed fry. However, no changes were found in the gonad somatic index (GSI; ovary weight ratio to total body weight); by the 7-month TBT treatment (2 µg/L) despite increasing GSI in controls, which suggested a lack of maturation of egg tissue and consequently a potential reduced fecundity (Holm et al. 1991). In sheepshead minnows, reduction in both total and percent viable eggs was found at more than 1.3 µg/L of TBT exposure, although the reductions were not statistically significant (Manning et al. 1999). TBT exposure to Japanese medaka at 1 mg/kg body weight caused a reduction of the spawning frequency (Nirmala et al. 1999). Additionally, environmentally relevant concentrations of