Hashimoto, Y., Moriguchi, Y., Oshima, H., Kawaguchi, M., Miyazaki, K., Nakamura, M., 2001. Measurement of estrogenic activity of chemicals for the development of new dental polymers. Toxicol. In Vitro 15, 421–425.

Hirata-Koizumi, M., Hamamura, M., Furukawa, H., Fukuda, N., Ito, Y., Wako, Y., et al., 2005a. Elevated susceptibility of newborn as compared with young rats to 2-*tert*-butylphenol and 2,4-di-*tert*-butylphenol. Congenit. Anom. (Kyoto) 45, 146–153.

Hirata-Koizumi, M., Kusuoka, O., Nishimura, N., Wada, H., Ogata, H., Fukuda, N., et al., 2005b. Susceptibility of newborn rats to hepatotoxicity of 1,3-dibromopropane and 1,1,2,2-tetrabromomethane, compared with young rats. J. Toxicol. Sci. 30, 29–42.

Horster, M., 1977. Nephron function and perinatal homeostasis. Ann. Rech. Vet. 8, 468–482.

IFCS, 2005. Chemical safety and children's health. Protecting the world's children from harmful chemical exposure. A global guide to resources. Prepared by the Intergovernmental Forum on Chemical Safety (IFCS) Children and Chemical Safety Working Group. World Health Organization, Geneva.

James, S.P., Pue, M.A., Richards, D.H., 1981. Metabolism of 1,3-dibromopropane. Toxicol. Lett. 8, 7–15.

Katsuda, S., Yoshida, M., Watanabe, G., Taya, K., Maekawa, A., 2000. Irreversible effects of neonatal exposure to *p-tert*-octylphenol on the reproductive tract in female rats. Toxicol. Appl. Pharmacol. 165, 217–226.

Kavlock, R.J., Rehnberg, B.F., Rogers, E.H., 1987. Critical prenatal period for chlorambucil-induced functional alterations of the rat kidney. Toxicology 43, 51–64.

Kawade, N., Onishi, S., 1981. The prenatal and postnatal development of UDP-glucuronyltransferase activity towards bilirubin and the effect of premature birth on this activity in the human liver. Biochem. J. 196, 257–260.

Kennedy, C.H., Cohen, K.B., Bechtold, W.E., Chang, I.Y., Eidson, A.F., Dahl, A.R., et al., 1993. Effect of dose on the metabolism of 1,1,2,2-tetrabromoethane in F344/N rats after gavage administration. Toxicol. Appl. Pharmacol. 119, 23–33.

Khan, S.A., Ball, R.B., Hendry 3rd, W.J., 1998. Effects of neonatal administration of diethylstilbestrol in male hamsters: disruption of reproductive function in adults after apparently normal pubertal development. Biol. Reprod. 58, 137–142.

Koizumi, M., Yamamoto, Y., Ito, Y., Takano, M., Enami, T., Kamata, E., et al., 2001. Comparative study of the toxicity of 4-nitrophenol and 2,4-dinitrophenol in newborn and young rats. J. Toxicol. Sci. 26, 299–311.

Koizumi, M., Nishimura, N., Enami, T., Kamata, E., Hasegawa, R., 2002. Comparative toxicity study of 3-aminophenol in newborn and young rats. J. Toxicol. Sci. 27, 411–421.

Koizumi, M., Noda, A., Ito, Y., Furukawa, M., Fujii, M., Kamata, E., et al., 2003. Higher susceptibility of newborn than young rats to 3-methylphenol. J. Toxicol. Sci. 28, 59–70.

Landrigan, P.J., Kimmel, C.A., Correa, A., Eskenazi, B., 2004. Children's health and the environment: public health issues and challenges for risk assessment. Environ. Health Perspect. 112, 257–265.

Lee, P.C., 1998. Disruption of male reproductive tract development by administration of the xenoestrogen, nonylphenol, to male newborn rats. Endocrine 9, 105–111.

Lo, S.C., Agar, N.S., 1986. NADH-methemoglobin reductase activity in the erythrocytes of newborn and adult mammals. Experientia 42, 1264–1265.

Manahan, S.E., 2003. Toxicological Chemistry and Biochemistry, third ed. Lewis publishers, Florida.

Merlet-Benichou, C., Gilbert, T., Muffat-Joly, M., Leliever-Pegorier, M., Leroy, B., 1994. Intrauterine growth retardation leads to a permanent nephron deficit in the rat. Pediatr. Nephrol. 8, 175–180.

MHLW, 2003. Single dose oral toxicity test of tetrabromoethane. Ministry of Health, Labour and Welfare (MHLW), Toxicity Testing Reports of Environmental Chemicals 10, pp. 45–46.

Moser, V.C., Padilla, S., 1998. Age- and gender-related differences in the time course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. Toxicol. Appl. Pharmacol. 149, 107–119.

OECD Test Guideline 407, Repeated-dose 28-day Oral Toxicity Study in Rodents. Adopted by the Council on 27th July 1995.

OECD Test Guideline 416, Two-Generation Reproduction Toxicity Study. Updated Guideline 22nd January 2001.

Orth, J.M., 1982. Proliferation of Sertoli cells in fetal and postnatal rats: A quantitative autoradiographic study. Anat. Rec. 203, 485–492.

Orth, J.M., 1984. The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology 115, 1248–1255.

Polin, R.A., Fox, W.W., Abman, S.H. (Eds.), 2004. Fetal and Neonatal Physiology, third ed. Saunders, Philadelphia.

Pope, C.N., Liu, J., 1997. Age-related differences in sensitivity to organophosphorus pesticides. Environ. Toxicol. Pharmacol. 4, 309–314.

Rachmel, A., Hazelton, G.A., 1986. The inducibility and ontogeny of rat liver UDP-glucuronyltransferase toward furosemide. Biochem. Pharmacol. 35, 3777–3782.

Rane, A., 1992. Drug disposition and action in infants and children. In: Yaffe, S.J., Aranda, J.V. (Eds.), Pediatric Pharmacology, Therapeutic Principles in Practice. Saunders, Philadelphia, pp. 10–21.

Renwick, A.G., 1998. Toxicokinetics in infants and children in relation to the ADI and TDI. Food Addit. Contam. 15 (Supplement), 17–35.

Rich, K.J., Boobis, A.R., 1997. Expression and inducibility of P450 enzymes during liver ontogeny. Microsc. Res. Technol. 39, 424–435.

Robinson, D., Smith, J.N., Williams, R.T., 1951. Studies in detoxication 39. Nitro compounds. (a) The metabolism of *o*-, *m*- and *p*-nitrophenols in the rabbit. (b) The glucuronides of the mononitrophenols and observations on the anomalous optical rotations of triacetyl β-*o*-nitrophenylglucuronide and its methyl ester. Biochem. J. 50, 221–227.

Scheuplein, R., Charnley, G., Dourson, M., 2002. Differential sensitivity of children and adults to chemical toxicity: I. Biological basis. Regul. Toxicol. Pharmacol. 35, 429–447.

Sheehan, D.M., Gaylor, D.W., 1990. Analysis of the adequacy of safety factors. Teratology 41, 590–591.

Stroheker, T., Chagnon, M.C., Pinnert, M.F., Berges, R., Canivenc-Lavier, M.C., 2003. Estrogenic effects of food wrap packaging xenoestrogens and flavonoids in female Wistar rats: a comparative study. Reprod. Toxicol. 17, 421–432.

Suzuki, A., Sugihara, A., Uchida, K., Sato, T., Ohta, Y., Katsu, Y., et al., 2002. Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. Reprod. Toxicol. 16, 107–116.

Takahashi, M., Ogata, H., Izumi, H., Ymashita, K., Takechi, M., Hirata-Koizumi, M., et al., 2004. Comparative study of toxicity of 2,4,6-trinitrophenol (picric acid) in newborn and young rats. Congenit. Anom. (Kyoto) 44, 204–214.

Takahashi, M., Hirata-Koizumi, M., Nishimura, N., Ito, Y., Sunaga, M., Fujii, S., et al., 2006. Susceptibility of newborn rats to 3-ethylphenol and 4-ethylphenol compared with that of young rats. Congenit. Anom. (Kyoto) 46, 26–33.

Tee, L.B., Gilmore, K.S., Meyer, D.J., Ketterer, B., Vandenberghe, Y., Yeoh, G.C., 1992. Expression of glutathione *S*-transferase during rat liver development. Biochem. J. 282, 209–218.

Toppari, J., Larsen, J.C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette Jr., L.J., et al., 1996. Male reproductive health and environmental xenoestrogens. Environ. Health Perspect. 104 (Suppl. 4), 741–803.

Trevisan, A., Rizzi, E., Scapinello, A., Gioffre, F., Chiesura, P., 1989. Liver toxicity due to 1,2-dichloropropane in the rat. Arch. Toxicol. 63, 445–449.

Watkins, J.B., Klaassen, C.D., 1985. Development of UDP-glucuronosyltransferase activity toward digitoxigenin-monodigitoxoside in neonatal rats. Drug Metab. Dispos. 13, 186–191.

Yamasaki, K., Takeyoshi, M., Yakabe, Y., Sawaki, M., Imatanaka, N., Takatsuki, M., 2002. Comparison of reporter gene assay and immature rat uterotrophic assay of twenty-three chemicals. Toxicology 170, 21–30.

Yoshida, T., Takehara, Y., Shimatani, M., Abe, K., Utsumi, K., 1982. Lipid peroxidation and antioxidants in rat liver during development. Tohoku J. Exp. Med. 137, 391–400.

Zoetemelk, C.E., Oei, I.H., van Meeteren Walchli, B., Onkenhout, W., van der Gen, A., Breimer, D.D., 1986. Biotransformation of 1,2-dibromopropane in rats into four mercapturic acid derivatives. Drug Metab. Dispos. 14, 601–607.

Early Pregnancy Failure Induced by Dibutyltin Dichloride in Mice

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Received 27 June 2006; revised 14 August 2006; accepted 1 September 2006

ABSTRACT: In this study, we examined the adverse effects of dibutyltin on initiation and maintenance of pregnancy after maternal administration during early pregnancy in mice. Following successful mating, female ICR mice were given dibutyltin dichloride (DBTCl) at 0, 7.6, 15.2, or 30.4 mg/kg bw/day by gastric intubation on days 0–3 or days 4–7 of pregnancy. Female mice were sacrificed on day 18 of pregnancy, and the pregnancy outcome was determined. After administration of DBTCl on days 0–3, the rate of non-pregnant females and the incidence of preimplantation embryonic loss were significantly increased at 30.4 mg/kg bw/day. The incidences of postimplantation embryonic loss in females given DBTCl on days 0–3 at 15.2 mg/kg and higher and on days 4–7 at 7.6 mg/kg bw/day and higher were increased. No increase in the incidence of fetuses with external malformations was observed after the administration of DBTCl on days 0–3 or days 4–7. A decline in the serum progesterone levels was detected in mice given DBTCl at 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The data show that DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice and suggest that the decline in serum progesterone levels is responsible for pregnancy failure. © 2007 Wiley Periodicals, Inc. Environ Toxicol 22: 44–52, 2007.

Keywords: dibutyltin dichloride; organotin; pregnancy failure; early embryonic loss; progesterone

INTRODUCTION

Organotin compounds are chemicals widely used in agriculture and industry. Disubstituted organotin compounds are commercially the most important derivatives, being used as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during the 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 (Piver, 1973; WHO, 1980). Wide-spread use of organotin compounds has caused increasing amounts to be released into environment. The most important route of entry of organotin compounds as nonpesticides into the environment is through the leaching of organotin-stabilized PVC by water (Quevauviller et al., 1991), and its use in antifouling agents resulting in the entry of organotin into the aquatic environment (Maguire, 1991). The identification of dibutyltin (DBT) and tributyltin (TBT) in aquatic marine organisms (Sasaki et al., 1988; Lau, 1991) and marine products (Suzuki et al., 1992) has been reported. TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT in the environment (Seligman et al., 1988; Stewart and de Mora, 1990). Food chain bioaccumulation 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) has been reported. These findings indicate that butyltins accumulate in the

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Contract grant sponsor: Ministry of Health, Labor and Welfare, Japan.

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/tox.20232

food chain and are bioconcentrated, and that humans can be exposed to butyltins via food.

Organotins possesses toxic effects on reproduction and development in experimental animals (Ema and Hirose, 2006). We previously reported that dibutyltin dichloride (DBTCl) by gavage throughout the period of organogenesis resulted in a significant increase in the incidence of fetal malformations in rats (Ema et al., 1991) and that rat embryos were highly susceptible to the teratogenic effects of DBTCl when administered on day 7 and day 8 of pregnancy (Ema et al., 1992). Tetrabutyltin (TeBT) is metabolized to TBT, DBT, and monobutyltin (MBT) derivatives (Fish et al., 1976; Kimmel et al., 1977). The TBT compound is metabolized to DBT and MBT derivatives and DBT is metabolized to MBT derivatives (Iwai et al., 1981). The developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from those of TeBT, TBT, and MBT in its mode of action, because the susceptible period for teratogenicity and types of malformations induced by DBT are different from those induced by TeBT, TBT, and MBT (Ema et al., 1995, 1996). Tributyltin chloride (TBTCl) (Harazono et al., 1996, 1998ab) and DBTCl (Ema and Harazono, 2000ab) during early pregnancy produced pregnancy failure in rats. In rats, the predominant adverse effects on reproduction and development of TBTCl and DBTCl on days 0–3 of pregnancy were a decrease in the pregnancy rate and an increase in the incidence of preimplantation embryonic loss, and TBTCl and DBTCl on days 4–7 of pregnancy mainly caused postimplantation embryonic loss (Harazono et al., 1998b; Ema and Harazono, 2000ab). The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl (Ema and Harazono, 2000b). Thus, the possibility exists that DBTCl and/or metabolites participate in the induction of early embryonic loss due to TBTCl.

The reproductive and developmental effects of organotin compounds, including DBT, were extensively investigated in rats (Ema and Hirose, 2006). We are unaware of any studies in which the adverse effects of DBT on initiation and maintenance of pregnancy have been assessed in mice. Studies in mice would be of great value in evaluating the reproductive and developmental toxicity of DBT. The present study was therefore conducted to determine the adverse effects on the initiation and maintenance of pregnancy of maternal exposure to DBTCl during early pregnancy in mice.

MATERIALS AND METHODS

Animal Husbandry and Maintenance

Male and female Crlj:CD1(ICR) mice at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, (Yokohama, Japan). The mice were acclimatized to the laboratory for 11 days prior to the start of the experiment. Male and female mice found to be in good health were selected for use. Female mice were caged with male mice and checked the following morning for signs of successful mating by examining vaginal plugs. The day when vaginal plugs were detected was considered to be day 0 of pregnancy. Successfully mated females were distributed into eight groups of 12 mice each and housed individually. Animals were reared on a γ-irradiated basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and filtered tap water *ad libitum*, and maintained in an air-conditioned room at 22°C ± 3°C, with a relative humidity of 50% ± 20%, under a controlled 12 h light/dark cycle, and ventilation with 10–15 air changes/hour. This study was performed in 2005 at the Safety Research Institute for Chemical Compounds. (Sapporo, Japan) in compliance with the "Law for the Humane Treatment and Management of Animals" (Ministry of the Environment, Japan, 1973), "Standards Relating to the Care and Management, etc. of Experimental Animals" (Prime Minister's Office, Japan, 1980) and "Guidance for Animal Care and Use of the Safety Research Institute for Chemical Compounds, Co."

Chemicals and Dosing

DBTCl was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The DBTCl used in this study was 99.5% pure, and it was kept in a dark and cool place. DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Osaka, Japan). The female mice were dosed once daily by gastric intubation with DBTCl at a dose of 7.6, 15.2, or 30.4 mg/kg bw (25, 50 or 100 μmol/kg bw) on days 0–3 of pregnancy or on days 4–7 of pregnancy. The dosage levels were determined based on the results of our previous studies, in which increases in the incidence of pre- and postimplantation embryonic loss were caused in female rats gavaged with DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 and days 4–7 of pregnancy, respectively (Ema and Harazono, 2000ab) and our dose-finding study in which no adverse effects on embryonic survival at 15.2 mg/kg bw/day and lower, increased embryonic loss at 30.4 mg/kg bw/day, and one death and three pregnancy failure in four females at 60.8 mg/kg bw/day were found in mice gavaged with DBTCl on days 0–3 of pregnancy. The volume of each dose was adjusted to 5 mL/kg of body weight based on the daily body weight. The control mice received olive oil only on days 0–3 or days 4–7 of pregnancy. All DBTCl solutions were prepared fresh daily.

Observations

All mice were observed for clinical signs of toxicity twice a day during the administration period and daily during the nonadministration period. Females showing a moribund condition were euthanized under ether anesthesia. Maternal

TABLE I. Maternal findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	1	0	0
Moribund condition (euthanized)	0	1	1	1
Vaginal discharge	0	1	0	0
Jaundice	0	2	7*	10*
Decreased locomotor activity	0	2	1	1
Hypothermia	0	1	1	1
Soil of perigenital fur	0	0	1	0
Initial body weight (g)[a]	27.4 ± 2.0	27.2 ± 2.1	27.2 ± 2.4	27.2 ± 2.1
Body weight gain (g)[a]				
Days 0–4	1.7 ± 1.1	0.6 ± 1.2	1.2 ± 1.6	0.3 ± 0.9*
Days 4–8	2.9 ± 1.5	2.5 ± 2.6	2.1 ± 2.0	1.6 ± 1.5
Days 8–18	20.1 ± 9.1	21.3 ± 12.4	13.6 ± 12.2	8.6 ± 12.2
Adjusted weight gain[b]	8.9 ± 3.4	9.9 ± 3.8	7.9 ± 4.8	5.3 ± 5.0
Food consumption (g)[a]				
Days 0–4	18.2 ± 1.8	15.0 ± 1.9*	16.7 ± 3.2	14.8 ± 2.3*
Days 4–8	22.9 ± 4.9	22.0 ± 2.7	21.7 ± 3.5	20.9 ± 3.5
Days 8–18	71.7 ± 10.1	71.0 ± 12.5	64.6 ± 13.3	57.8 ± 13.4*

[a] Values are given as mean ± SD.
[b] Adjusted weight gain refers to body weight gain excluding the uterus.
* Significantly different from the control, $P < 0.05$.

body weight was recorded daily, and food consumption was recorded on days 0, 4, 8, 12, and 18 of pregnancy. The females were euthanized by exsanguination under ether anesthesia on day 18 of pregnancy. The uterus was weighed and the number of corpora lutea was recorded. The numbers of implantations, live and dead fetuses, and of resorptions were counted. The uteri were placed in 10% ammonium sulfide for confirmation of the dam's pregnancy status (Salewski, 1964). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. The placental weight was also measured.

Analysis of Serum Steroids Hormone Levels

Blood samples were collected from the abdominal aorta under ether anesthesia on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl at 0 or 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The serum was separated and stored at −80°C for later assay of steroid hormones. Serum progesterone and 17β-estradiol were measured by Teizo Medical (Kawasaki, Japan) using the liquid chromatography-electrospray ionization Tandem Mass Spectrometry (LC-MS/MS, Applied Biosystems/MDS SCIEX). The detection limits of serum progesterone and 17β-estradiol were 10.0 and 0.25 pg/mL, respectively. The intra- and interassay coefficients of variation for 17β-estradiol were below 6.4% and 8.9%, respectively. The intra- and interassay coefficients of variation for progesterone were below 9.0% and 7.9%, respectively.

Statistical Analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, food consumption, numbers of corpora lutea, implantations, embryonic/fetal loss and live fetuses, fetal weight, and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney U test. The incidences of pre- and postimplantation embryonic loss and fetuses with external malformations were analyzed using Wilcoxon's rank sum test. The incidence of clinical signs in dams, pregnancy, nonpregnancy, and litters with fetal malformations, and the sex ratio of live fetuses were analyzed using Fisher's exact test. The levels of serum progesterone and 17β-estradiol were analyzed by Student's t-test. The 0.05 level of probability was used as the criterion for significance.

TABLE II. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	3	4	7*
No. of pregnant females	11	9	8	5*
No. of implantations per female[a,b]	9.5 ± 5.1	9.8 ± 7.1	8.3 ± 7.0	5.4 ± 6.7
Pre-implantation loss per female (%)[a,b]	9.7	29.7[c]	34.0	58.3*
No. of pregnant females surviving until scheduled sacrifice	11	8	7	4
No. of litters totally resorbed	0	0	1	1
No. of corpora lutea per litter[a,d]	10.5 ± 4.3	13.1 ± 4.9	12.4 ± 4.4	13.3 ± 1.3
No. of implantations per litter[a,d]	10.4 ± 4.3	12.6 ± 4.9	12.3 ± 4.4	13.3 ± 1.3
Pre-implantation loss per litter (%)[d,e]	1.5	3.3	1.1	0
No. of post-implantation loss per litter[a,d]	1.0 ± 1.0	1.1 ± 1.5	4.1 ± 3.2	4.0 ± 5.4
Post-implantation loss per litter (%)[d,f]	10.1	14.1	41.3*	32.2
No. of live fetuses per litter[a,d]	9.4 ± 4.2	11.5 ± 5.3	8.1 ± 5.0	9.3 ± 6.2
Sex ratio of live fetuses (male / female)	50/53	47/45	30/27	21/16
Body weight of live fetuses (g)[a]				
Male	1.54 ± 0.19	1.30 ± 0.12*	1.14 ± 0.22*	1.12 ± 0.10*
Female	1.42 ± 0.15	1.28 ± 0.20	1.08 ± 0.26*	1.01 ± 0.11*
External examinations of fetuses				
No. of fetuses (litters) examined	103 (11)	92 (8)	57 (6)	37 (3)
No. of fetuses (litters) with anomalies	1 (1)	0	1 (1)	0
Cleft palate	1	0	1	0
Kinked tail	0	0	1	0
Placental weight (mg)[a]	125 ± 56	116 ± 15	120 ± 17	119 ± 16

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

RESULTS

Administration of DBTCl on Days 0–3 of Pregnancy

Table I shows the maternal findings in mice given DBTCl on days 0–3 of pregnancy. One death was observed at 7.6 mg/kg bw/day, and one female each showed a moribund condition at 7.6, 15.2, and 30.4 mg/kg bw/day, and was euthanized. The female mice in the DBTCl-treated groups showed vagina discharge, jaundice, decreased locomotor activity, hypothermia and/or soiled perigenital fur, and the incidence of females showing jaundice was significantly increased at 15.2 mg/kg bw/day and higher. A significantly decreased body weight gain on days 0–4 was noted at 30.4 mg/kg bw/day. Food consumption on days 0–4, days 4–8, and days 8–18 in the DBTCl-treated groups were reduced, and significantly decreased food consumptions on days 0–4 at 7.6 and 30.4 mg/kg bw/day and on days 8–18 at 30.4 mg/kg bw/day were observed.

The reproductive and developmental findings in mice given DBTCl on days 0–3 of pregnancy are shown in Table II. The total absence of any implantation site, i.e., nonpregnancy, was found in one, three, four, and seven of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively. In the successfully mated females, the pregnancy rate was significantly decreased, and the incidence of preimplantation embryonic loss per females was significantly increased at 30.4 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, the number of corpora lutea per litter, implantations per litter, live fetuses per litter, the incidence of litters totally resorbed and of preimplantation loss per litter, and the sex ratio of live fetuses were not significantly different between the control and DBTCl-treated groups. The incidence of postimplantation loss per litter was increased in the DBTCl-treated groups, and a significant increase was observed at 15.2 mg/kg bw/day. A significantly lower fetal weight was found in males at 7.6 mg/kg bw/day and in both sexes at 15.2 and 30.4 mg/kg bw/day. One fetus with cleft palate in the control group and one fetus with a cleft palate and kinked tail in the 15.2 mg/kg bw/day group were observed. The placental weight in the DBTCl-treated

TABLE III. Maternal findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	0	1	0
Moribund condition (euthanized)	0	0	0	1
Vaginal discharge	0	0	4	4
Jaundice	0	0	2	6*
Decreased locomotor activity	0	0	0	1
Hypothermia	0	0	0	1
Initial body weight (g)[a]	28.1 ± 1.8	28.1 ± 1.8	28.1 ± 1.8	28.2 ± 1.7
Body weight gain (g)[a]				
Days 0–4	1.6 ± 1.0	1.9 ± 0.8	1.2 ± 1.2	1.6 ± 0.9
Days 4–8	3.1 ± 1.1	1.9 ± 1.6	0.5 ± 1.8*	−0.3 ± 2.1*
Days 8–18	24.9 ± 9.1	14.9 ± 8.9*	2.9 ± 6.3*	2.4 ± 2.4*
Adjusted weight gain[b]	8.3 ± 3.5	8.1 ± 4.3	3.2 ± 5.3*	3.8 ± 3.2*
Food consumption (g)[a]				
Days 0–4	18.5 ± 1.9	18.9 ± 2.4	18.4 ± 2.7	18.8 ± 1.3
Days 4–8	21.8 ± 1.9	19.2 ± 2.6	16.4 ± 3.3*	15.6 ± 3.5*
Days 8–18	74.5 ± 12.1	67.7 ± 9.9	55.2 ± 12.6*	57.2 ± 6.2*

[a] Values are given as mean ± SD.
[b] Adjusted weight gain refers to body weight gain excluding the uterus.
* Significantly different from the control, $P < 0.05$.

groups was not significantly different from that in the control group.

Administration of DBTCl on Days 4–7 of Pregnancy

Table III shows the maternal findings in mice given DBTCl on days 4–7 of pregnancy. One death was observed at 15.2 mg/kg bw/day, and one female that showed a moribund condition at 30.4 mg/kg bw/day was euthanized. The female mice in the DBTCl-treated groups showed vaginal discharge, jaundice, decreased locomotor activity, and/or hypothermia, and the incidence of females with jaundice was significantly increased at 30.4 mg/kg bw/day. The body weight gain on days 4–8 and adjusted weight gain, which indicates the net weight gain of female mice, at 15.2 mg/kg bw/day and higher, and on days 8–18 at 7.6 mg/kg bw/day and higher were significantly decreased. Food consumption on days 4–8 and days 8–18 was significantly lowered at 15.2 mg/kg bw/day and higher.

The reproductive and developmental findings in mice given DBTCl on days 4–7 of pregnancy are presented in Table IV. Although nonpregnancy was found in one, two, and one of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively, no significant decrease in the pregnancy rate was noted in the DBTCl-treated groups. In the successfully mated females, the number of implantations per female was significantly decreased at 15.2 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, totally resorbed litters were found in 2 of the 11 females at 7.6 mg/kg bw/day, 8 of the 9 females at 15.2 mg/kg bw/day, and 10 of the 10 females at 30.4 mg/kg bw/day. At 30.4 mg/kg bw/day, no live fetuses were obtained. The numbers of corpora lutea per litter, implantations per litter, and preimplantation loss per litter, and the sex ratio of live fetuses in the DBTCl-treated groups were not significantly different from those in the control group. A significant increase in the number and incidence of postimplantation loss per litter, and a decrease in the number of live fetuses were found in the DBTCl-treated groups. The weights of male and female fetuses were significantly lowered at 7.6 mg/kg bw/day. One fetus with omphalocele, and one fetus with exencephaly and open eyelids were observed at 7.6 mg/kg bw/day. The placental weight was not significantly different between the control and the DBTCl-treated groups.

Serum Progesterone and 17β-Estradiol Levels

The serum progesterone and 17β-estradiol levels are shown in Figure 1. A significant reduction in the serum progesterone levels was noted in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Although higher levels of serum 17β-estradiol were observed after the administration of DBTCl on days 4–7 of pregnancy, no statistically significant difference in 17β-estradiol levels were detected between the control and DBTCl-treated groups.

DISCUSSION

The present study was designed to evaluate the adverse effects of DBTCl on the initiation and maintenance of

TABLE IV. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	1	2	1
No. of pregnant females	11	11	10	11
No. of implantations per female[a,b]	12.6 ± 4.4	13.2 ± 4.6	7.5 ± 5.7*	11.1 ± 5.4
Pre-implantation loss per female (%)[a,b]	8.9	8.9	24.7	18.3[c]
No. of pregnant females surviving until scheduled sacrifice	11	11	9	10
No. of litters totally resorbed	0	2	8*	10*
No. of corpora lutea per litter[a,d]	13.8 ± 2.1	14.5 ± 2.3	10.6 ± 5.2	13.9 ± 2.8
No. of implantations per litter[a,d]	13.7 ± 2.1	14.4 ± 2.2	9.4 ± 5.1	12.7 ± 4.1
Pre-implantation loss per litter (%)[d,e]	0.6	0.6	10.7	10.2
No. of postimplantation loss per litter[a,d]	0.6 ± 1.0	7.2 ± 6.1*	8.7 ± 4.8*	12.7 ± 4.1*
Post-implantation loss per litter (%)[d,f]	4.3	48.3*	94.4*	100*
No. of live fetuses per litter[a,d]	13.1 ± 2.0	7.2 ± 5.6*	0.8 ± 2.3*	0
Sex ratio of live fetuses (male/female)	82/62	50/29	4/3	
Body weight of live fetuses (g)[a]				
Male	1.45 ± 0.10	1.23 ± 0.10*	1.27	
Female	1.39 ± 0.10	1.18 ± 0.14*	1.18	
External examinations of fetuses				
No. of fetuses (litters) examined	144 (11)	79 (9)	7 (1)	
No. of fetuses (litters) with anomalies	0	2 (2)	0	
Omphalocele	0	1	0	
Exencephaly and open eyelids	0	1	0	
Placental weight (mg)[a]	102 ± 10	99 ± 12	114	

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

pregnancy following maternal exposure during early pregnancy in mice. The most striking finding in the present study is pregnancy failure, decrease in the pregnancy rate, and litters totally resorbed, in females given DBTCl during early pregnancy.

Death and/or moribund condition were observed after the administration of DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 of pregnancy and at 15.2 mg/kg bw/day and higher on days 4–7 of pregnancy, and significant increased incidence of females showing clinical signs of toxicity were found after the administration of DBTCl at 15.2 mg/kg bw/day and higher on days 0–3 of pregnancy and at 30.4 mg/kg bw/day on days 4–7 of pregnancy. These findings indicate that more severe general toxicity was induced by DBTCl on days 0–3 of pregnancy than that on days 4–7 of pregnancy. However, adverse effects on body weight gain were detected after the administration of DBTCl at 30.4 mg/kg bw/day on days 0–3 of pregnancy and at 7.6 mg/kg bw/day and higher on days 4–7 of pregnancy. Although the recovery of body weight gain was observed after the administration of DBTCl on days 0–3 of pregnancy, recovery by the end of the study was not found in females given DBTCl at 7.6 mg/kg bw/day and higher after the administration on days 4–7 of pregnancy. Following the administration on days 4–7 of pregnancy, a significantly lower adjusted weight gain was also noted in females given DBTCl at 15.2 mg/kg/day and higher. These findings indicate that more severe adverse effects on body weight gain were induced by DBTCl on days 4–7 of pregnancy than that on days 0–3 of pregnancy. More severe effects of DBTCl on body weight gain following the administration on days 4–7 may be attributable to the significant decrease in the number of live fetuses.

The earlier administration period, days 0–3 of pregnancy, corresponds to the period before implantation, and the later administration period, days 4–7 of pregnancy, corresponds to the period when implantation is in progress and the period shortly after implantation in mice (Rugh, 1968). We expected that DBTCl insult on days 0–3 of pregnancy might result in preimplantation loss of embryos; i.e., the absence or decrease of implantation sites, and DBTCl insult on days 4–7 of pregnancy might result in postimplantation loss of embryos; i.e.,

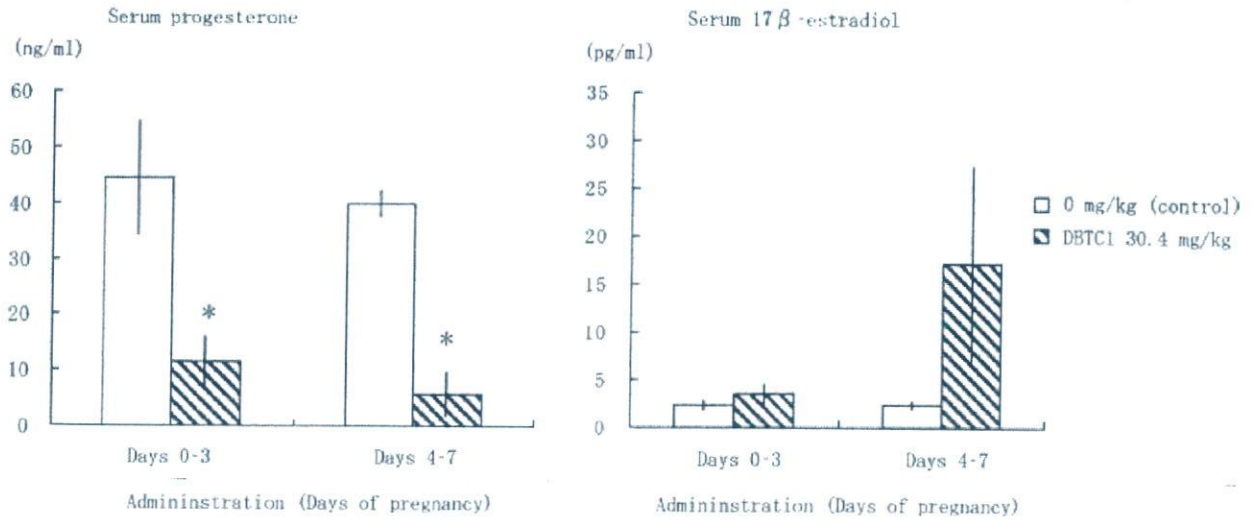

Fig. 1. Serum progesterone and 17β-estradiol levels in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Blood samples were collected on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± SEM of seven or eight mice. *Significantly different from the control group, $P < 0.05$.

the resorption of implantation sites. The most striking adverse effects of DBTCl on reproduction and development were a decrease in pregnancy rate, complete implantation failure, when DBTCl was given to mice on days 0–3 of pregnancy. The findings of an increased incidence of preimplantation embryonic loss in successfully mated females, and an increased incidence of postimplantation embryonic loss and low fetal weight in pregnant females survived until scheduled sacrifice after the administration of DBTCl on days 0–3 of pregnancy may suggest that DBTCl adversely affects preimplantation embryos and also the later survival and growth of embryos/fetuses when administered during the preimplantation period. On the other hand, the predominant adverse effects of DBTCl on reproduction and development were postimplantation loss, complete litter loss, when DBTCl was given to mice on days 4–7 of pregnancy. The findings of an increase in the incidence of postimplantation embryonic loss and a decrease in the fetal weight after administration of DBTCl on days 4–7 of pregnancy may suggest that DBTCl has effects on the later survival and growth of embryos/fetuses when administered during the peri-implantation period. Considered collectively, these findings indicate that the manifestation of adverse effects of DBTCl on reproduction and development varies with the stages of pregnancy at the time of maternal exposure.

The corpora lutea are essential up to the end of pregnancy in mice (Deansely, 1966). The embryo transport process in mice is triggered by progesterone and requires progesterone activity for its maintenance (Kendle and Lee, 1980). In mice, 24 h of progesterone priming is not only adequate for implantation, but this priming has a long-term effect on implantation (Huet-Hudson and Dey, 1990). In our previous studies in rats, increases in the incidences of early embryonic loss were observed after the administration of DBTCl during early pregnancy (Ema and Harazono, 2000ab). The suppression of uterine decidualization and reduced levels of serum progesterone were found in female rats given DBTCl on days 0–3 or days 4–7 of pseudopregnancy (Harazono and Ema, 2003), and lowered reproductive parameters in female rats given DBTCl were recovered by the administration of progesterone (Ema et al., 2003). Based on these findings, we hypothesized that the decline in serum progesterone levels in pregnant animals was a primary mechanism for the implantation failure due to DBTCl in rats. In the present study in mice, a decline in serum progesterone levels was detected after the administration of DBTCl during early pregnancy. These findings are in good agreement with previous findings that DBTCl induced early embryonic loss and decreased serum progesterone levels in pregnant rats. There is a similarity in the effects of DBTCl on progesterone levels in early pregnancy in rats and mice, and these suggest that the decline in the serum progesterone levels is also the factor responsible for the DBTCl-induced pregnancy failure in mice. Early pregnancy failure was also caused by systemic activation of the CD-40 immune costimulatory pathway in mice (Erlebacher et al., 2004). They noted that pregnancy failure resulted from impaired progesterone synthesis by the corpus luteum of the ovary, an endocrine defect in turn associated with ovarian resistance to the gonadotropic effects of prolactin and that pregnancy failure also required the proinflammatory cytokine TNF-a and correlated with the luteal induction of the prolactin receptor signaling inhibitors suppressor of cytokine signaling 1

(Socs1) and Socs3. Our results of the present study may support their argument. To further evaluate the adverse effects of DBTCl during early pregnancy, determination of the gene expression profile in the uterus of mice and rats is currently in progress.

In conclusion, DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice, and the present data suggest that the decline in progesterone is the responsible factor for the early pregnancy failure in mice.

REFERENCES

Deansely R. 1966. The endocrinology of pregnancy and foetal life. In: Parks AS, editor. Marshall's Physiology of Reproduction, Vol. 3. Boston: Little Brown. pp 891–1063.

Ema M, Harazono A. 2000a. Adverse effects of dibutyltin dichloride on initiation and maintenance of rat pregnancy. Reprod Toxicol 14:451–456.

Ema M, Harazono A. 2000b. Developmental and reproductive toxicity of tributyltin and its metabolite, dibutyltin, in rats. Congenit Anom (Kyoto) 40:108–120.

Ema M, Hirose A. 2006. Reproductive and developmental toxicity of organotin compounds. In: Golub MS, editor. Metals, Fertility, and Reproductive Toxicity. New York: CRC Press (Taylor & Francis Group). pp 23–64.

Ema M, Itami T, Kawasaki H. 1991. Teratogenicity of di-*n*-butyltin dichloride in rats. Toxicol Lett 58:347–356.

Ema M, Itami T, Kawasaki H. 1992. Susceptible period for the teratogenicity of di-*n*-butyltin dichloride in rats. Toxicology 73: 81–92.

Ema M, Kurosaka R, Amano H, Ogasawa Y. 1995. Comparative developmental toxicity of butyltin trichloride, dibutyltin dichloride and tributyltin chloride in rats. J Appl Toxicol 15:297–302.

Ema M, Kurosaka R, Amano H, Ogawa Y. 1996. Comparative developmental toxicity of di-, tri-, and tetrabutyltin compounds after administration during late organogenesis in rats. J Appl Toxicol 16:71–76.

Ema M, Harazono A, Hirose A, Kamata E. 2003. Protective effects of progesterone on implantation failure induced by dibutyltin dichloride in rats. Toxicol Lett 143:233–238.

Erlebacher A, Zhang D, Parlow AF, Glimcher LH. 2004. Ovarian insufficiency and early pregnancy loss induced by activation of the innate immune system. J Clin Invest 114:39–48.

Evans DW, Laughlin RB Jr. 1984. Accumulation of bis(tributyltin)oxide by the mud crab, *Rhithropanopeus harrisii*. Chemosphere 13:213–219.

Fish RH, Kimmel EC, Casida JE. 1976. Bioorganotin chemistry: Reactions of tributyltin derivatives with a cytochrome P-450 dependent monooxygenese enzyme system. J Organomet Chem 118:41–54.

Harazono A, Ema M. 2003. Suppression of decidual cell response induced by dibutyltin dichloride in pseudopregnant rats: As a cause of early embryonic loss. Reprod Toxicol 17:393–399.

Harazono A, Ema M, Ogawa Y. 1996. Pre-implantation embryonic loss induced by tributyltin chloride in rats. Toxicol Lett 89:185–190.

Harazono A, Ema M, Kawashima K. 1998a. Evaluation of malnutrition as a cause of tributyltin-induced pregnancy failure in rats. Bull Environ Contam Toxicol 61:224–230.

Harazono A, Ema M, Ogawa Y. 1998b. Evaluation of early embryonic loss induced by tributyltin chloride in rats: Phase- and dose dependent antifertility effects. Arch Environ Contam Toxicol 34:94–99.

Huet-Hudson YM, Dey SK. 1990. Requirement for progesterone priming and its long-term effects on implantation in the mouse. Proc Soc Exp Biol Med 193:259–263.

Iwai H, Wada O, Arakawa Y. 1981. Determination of tri-, di-, and monobutyltin and inorganic tin in biological materials and some aspects of their metabolism in rats. J Anal Toxicol 5: 300–306.

Kannan K, Corsolini S, Focardi S, Tanabe S, Tatsukawa R. 1996. Accumulation pattern of butyltin compounds in dolphin, tuna, and shark collected from Italian coastal waters. Arch Environ Contam Toxicol 31:19–23.

Kendle KE, Lee B. 1980. Investigation of the influence of progesterone on mouse embryo transport by using antiprogestational steroids. J Reprod Fertil 58:253–258.

Kimmel EC, Fish RH, Casida JE. 1977. Bioorganotin chemistry. Metabolism of organotin compounds in microsomal momooxygenese system and in mammals. J Agric Food Chem 25:1–9.

Lau MM. 1991. Tributyltin antifoulings: A threat to the Hong Kong marine environment. Arch Environ Contam Toxicol 20: 299–304.

Laughlin RB Jr, French W, Guard HE. 1986. Accumulation of bis(tributyltin) oxide by the marine mussel *Mytilus edulis*. Environ Sci Technol 20:884–890.

Maguire RJ. 1991. Aquatic environmental aspects of nonpesticidal organotin compounds. Water Pollut Res J Can 26:243–260.

Ministry of the Environment. 1973. Law for the Humane Treatment and Management of Animals, Law No. 105, October 1, 1973, revised June 15, 2005. Japan: Ministry of the Environment.

Piver WT. 1973. Organotin compounds: Industrial applications and biological investigation. Environ Health Perspect 4:61–79.

Prime Minister's Office. 1980. Standards Relating to the Care and Management, etc. of Experimental Animals, Notification No. 6, March 27, 1980 of the Prime Minister's Office. Japan: Prime Minister's Office.

Quevauviller P, Bruchet A, Donard OFX. 1991. Leaching of organotin compounds from poly (vinyl chloride) (PVC) materials. Appl Organomet Chem 5:125–129.

Rugh R. 1968. The Mouse: Its Reproduction and Development. Minneapolis: Burgess Publishing. 85p.

Salewski E. 1964. Färbemethode zum makroskopischen nachweis von implantationssetellen am uterus der ratte. Naunyn-Schm Arch Exp Pathol Pharmakol 247:367–372.

Sasaki K, Ishizaka T, Suzuki T, Saito Y. 1988. Determination of tri-*n*-butyltin and di-*n*-butylin compounds in fish by gas chromatography with flame photometric detection. J Assoc Off Anal Chem 71:360–366.

Seligman PF, Valkirs AO, Stang PM, Lee RF. 1988. Evidence for rapid degradation of tributyltin in a marina. Mar Pollut Bull 19:531–534.

Short JW, Thrower FP. 1986. Accumulation of butyltins in muscle tissue of Chinook salmon reared in sea pens treated with tri-*n*-butyltin. Mar Pollut Bull 17:542–545.

Stewart C, de Mora SJ. 1990. A review of the degradation of tri (*n*-butyl) tin in the marine environment. Environ Technol 11:565–570.

Suzuki T, Matsuda R, Saito Y. 1992. Molecular species of tri-*n*-butyltin compounds in marine products. J Agric Food Chem 40:1437–1443.

Waldock MJ, Thain JE. 1983. Shell thickening in Crassostea gigas: Organotin antifouling or sediment induced? Mar Pollut Bull 14:411–415.

WHO. 1980. Environmental Health Criteria 15 Tin and Organotin Compounds: A Preliminary Review. Geneva: World Health Organization.

Drug and Chemical Toxicology, 29:215–225, 2006

ISSN: 0148-0545 print
DOI: 10.1080/01480540600566816

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Evaluation of Developmental Toxicity of Ultraviolet Absorber 2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-Chlorobenzotriazole in Rats

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2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as a UV absorber. In this study, the developmental toxicity of DBHCB was evaluated in rats. Pregnant rats were given DBHCB at 0, 62.5, 250, or 1000 mg kg^{-1} day^{-1} by gavage on days 5–19 of pregnancy. No deaths were observed in the pregnant rats of any group. No effect of DBHCB on the general conditions, body weight gain, or feed consumption was observed in the pregnant rats. There were no changes in the ovarian weight, gravid uterine weight, or necropsy findings in the maternal rats of the DBHCB-treated groups. No significant effects of DBHCB were found in the number of corpora lutea, implantations, live fetuses, resorptions or dead fetuses, incidence of pre- or postimplantation embryonic loss, viability of fetuses, fetal weight, or sex ratio of live fetuses. No significant difference in the incidence of fetuses with malformations or variations or degree of ossification was detected between the DBHCB-treated and control groups.

Keywords Benzotriazole, Developmental toxicity, Rat, UV absorber.

INTRODUCTION

2-(3',5'-Di-*tert*-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole (CAS no. 3864 99–1; DBHCB) is slightly yellowish powder, stable under ordinary conditions, and insoluble in water. Its melting point is 154–158°C, and its specific gravity

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is 1.26. This chemical provides effective light stabilization and prevents the yellowing and degradation of polymers such as polypropylene, high-density polyethylene, unsaturated polyester, styrene-based thermoplastics elastomer, polyamide and impact polystyrene and is used as a UV absorber (Chemical Land21, 2005). The finished polymers—which contain UV absorbers at levels not to exceed 0.5% by weight of polyethylene phthalate polymers, complying with 21 CFR 177.1630 (FDA, 2005a)—may be used in contact with some food types and used under certain conditions as described in 21 CFR 176.170 (FDA, 2000; 2005b). UV absorbers are used in food packages as plastic additives, their function being mainly to prevent polymer degradation and/or a change in the quality of the packed food due to UV rays.

It has caused some anxiety that humans have been exposed to these chemicals in occupational surroundings, from environmental contamination and from contamination in food migrated from packages. The possibility of these chemicals entering the biological system has aroused great concern about their toxic potential. Important information can be gained by studying the biological effects produced by environmental chemicals in laboratory animals, in order to investigate their possible influences on human health.

Recently, DBHCB was assessed for its estrogenic activity, using a recombinant yeast assay (Miller et al., 2001) and the yeast two-hybrid assay (Kawamura et al., 2003); it was reported that DBHCB was not estrogenic. Some information on toxicity is available (Everlight Chemical Industrial Corporation, 2002). The oral LD_{50} for DBHCB was greater than 5000 mg/kg in rats. DBHCB caused minimal irritation to the skin and slight irritation to the eyes in rabbits. A 90-day feeding study of DBHCB in rats, at 22–800 mg/kg, resulted in dose-dependent increases in liver weights and signs of liver toxicity. No effects were found at 3.7 mg/kg. However, no detailed information is available for the toxicity studies.

Although testing for reproductive and developmental toxicity has become an important part of the overall toxicology profile for chemicals, no information has yet been presented on the reproductive and developmental toxicity of DBHCB. Therefore, the current study was conducted to evaluate the developmental toxicity of DBHCB given orally to rats during pregnancy.

MATERIALS AND METHODS

This study was performed in compliance with the OECD Guideline 414 Prenatal Developmental Toxicity Study (OECD, 2001) in 2004 at the Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan).

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 11 weeks of age and females at 10 weeks of age were purchased from Hino Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for 1 week prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared with a basal diet (CE-2; Clea Co., Ltd., Tokyo, Japan), water was provided *ad libitum*, and the animals were maintained in an air-conditioned room at 21.6–22.2°C, with a relative humidity of 45–58%, a 12-h light/dark cycle, and ventilation with 15 air changes/hour. Virgin female rats were mated overnight with male rats. The day when the sperm and/or vaginal plug was found to be day 0 of pregnancy. The copulated females, weighing 245–314 g, 11 weeks old, were distributed on a random basis into 4 groups of 20 rats each and housed individually. This experiment was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the committee of SNBL.

Chemicals and Dosing

DBHCB was obtained from Musashino Geigy Co., Ltd. (Kitaibaraki, Japan). The DBHCB (lot no. 05004IX3) used in this study was 99.9% pure based on HPLC analysis, 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 the study. Rats were treated once daily by gastric intubation with DBHCB at a dosage of 0 (control), 62.5, 250, or 1000 mg/kg on day 5 through day 19 of pregnancy. The dosage levels were determined based on the results of our dose-finding study in which a significantly increased liver weight was caused in males at 250 mg kg^{-1} day^{-1} and higher, but not in females even at 1000 mg kg^{-1} day^{-1}, after administration of DBHCB for 14 days in rats. DBHCB was suspended in 5% gum arabic solution. The volume of each dose was adjusted to 10 mL/kg body weight based on the latest body weight. The control rats were given only 5% gum arabic solution. The stability of the formulations in a dark and cool place under airtight conditions had been confirmed for up to 14 days. During use, the formulations were maintained under such conditions for no more than 7 days and were 97.3% to 100.1% of the target concentration.

Observations

All females were observed daily during the preadministration period and twice a day (before administration and 1 to 2 h after administration) during the administration period for clinical signs of toxicity. Maternal body weight was recorded on days 0, 5, 8, 11, 14, 17, 19, and 20 of pregnancy. Feed consumption was recorded on days 0–1, 5–6, 8–9, 11–12, 14–15, 17–18, and 19–20

of pregnancy. The pregnant rats were euthanized by exsanguination under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the uterus and ovaries were removed from the maternal body and weighed. The numbers of corpora lutea, implantation sites, and live and dead fetuses and resorptions were counted. The live fetuses were removed from the uterus and sexed, weighed, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected, 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 free-hand razor-blade sectioning (Wilson, 1973), and the thoracic areas were subjected to microdissecting (Nishimura, 1974) to reveal internal abnormalities.

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 feed consumption of the pregnant rats, numbers of corpora lutea, implantations and live fetuses per litter, and fetal weight were analyzed with Bartlett's test (Snedecor and Cochran, 1974) for homogeneity of variance at the 5% level of significance. When the variance was homogeneous, Dunnett's test (Dunnett, 1996) was performed to compare the mean value in the control group with that in each DBHCB group. When the variance was heterogeneous, a Dunnett-type test (Miller, 1987) was performed to compare the mean value in the control group with that in each DBHCB group after rank conversion. 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 DBHCB and that of the control group. The incidence of dams with anomalous fetuses was analyzed with Fisher's exact test.

RESULTS

Table 1 shows the maternal findings in rats given DBHCB on days 5–19 of pregnancy. No deaths or clinical signs of toxicity were found in female rats of any group. There was no difference in the fertility rate between the control and DBHCB-treated groups. No effects of DBHCB on body weight gains on days 0–5, 5–14, 14–19, and 19–20 of pregnancy were observed. During the whole period of pregnancy, no effects of DBHCB were also detected in body weight gain. There was no difference in feed consumption during pregnancy between the control and DBHCB-treated groups. No effects of DBHCB on weights of the gravid uterus and ovaries were detected.

Table 1: Maternal findings in rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)			
	0 (control)	**62.5**	**250**	**1000**
No. of rats	20	20	20	20
No. of pregnant rats	17	18	17	18
No. of dead rats	0	0	0	0
Initial body weight	285 ± 11	280 ± 12	285 ± 18	288 ± 11
Body weight gain during pregnancy (g)[a]				
Days 0–5	30 ± 8	33 ± 5	31 ± 6	30 ± 6
Days 5–14	47 ± 7	44 ± 7	49 ± 5	43 ± 9
Days 14–19	71 ± 9	65 ± 10	67 ± 10	63 ± 12
Days 19–20	16 ± 6	17 ± 4	20 ± 5	18 ± 5
Days 0–20	163 ± 17	159 ± 19	167 ± 14	154 ± 20
Adjusted weight gain[b]	88 ± 9	88 ± 10	91 ± 10	82 ± 18
Feed consumption during pregnancy (g/day)[a]				
Days 0–1	24 ± 3	23 ± 3	23 ± 3	24 ± 4
Days 5–6	27 ± 3	27 ± 3	27 ± 3	27 ± 3
Days 8–9	28 ± 4	28 ± 3	28 ± 3	28 ± 2
Days 11–12	29 ± 4	29 ± 3	28 ± 2	29 ± 3
Days 14–15	28 ± 4	28 ± 3	28 ± 3	28 ± 3
Days 17–18	32 ± 4	30 ± 4	31 ± 3	31 ± 4
Days 19–20	29 ± 4	29 ± 3	31 ± 4	30 ± 3
Weight of gravid uterus (g)[a]	88 ± 9	88 ± 10	91 ± 10	82 ± 18
Weight of ovaries (mg)[a]	149 ± 21	137 ± 14	149 ± 19	139 ± 14

[a]Values are given as the mean ± SD.
[b]Adjusted weight gain refers to maternal weight gain excluding the gravid uterus.

The reproductive findings in rats given DBHCB on days 5–19 of pregnancy are presented in Table 2. No totally resorbed litters were found in any group. No effects of DBHCB were observed on the number of corpora lutea or implantations, incidence of pre- or postimplantation loss, or the number of live fetuses or the sex ratio of live fetuses. There was no difference in the body weight of male and female fetuses between the control and DBHCB-treated groups. No abnormal findings were noted in the placentae of any group.

Morphological findings in the live fetuses of rats given DBHCB on days 5–19 of pregnancy are shown in Table 3. No fetuses with external malformations were observed in any group. Skeletal examination revealed no fetuses with skeletal malformations in any group. Fetuses with skeletal variations were observed in all groups including the control group. The incidence of fetuses with individual skeletal variations was not increased after the administration of DBHCB. The total number of fetuses with skeletal variations was also not increased in the DBHCB-treated groups. The degree of ossification, as evidenced by the numbers of sacral and caudal vertebrae and sternebrae in the DBHCB-treated groups, was not different from that in the control group. No fetuses with internal malformations were detected in any group. The fetuses with internal variations, such as thymic remnants in the neck, dilated renal

Table 2: Reproductive findings in rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)				
	0 (control)	**62.5**	**250**	**1000**	**Historical control values[d]**
No. of litters	17	18	17	18	652 (48 studies)
No. of litters totally resorbed	0	0	0	0	
No. of corpora lutea per litter[a]	16.9 ± 2.0	16.3 ± 1.1	17.1 ± 1.7	16.6 ± 1.9	13.8–17.5
No. of implantations per litter[a]	16.2 ± 1.4	15.8 ± 1.1	16.6 ± 1.6	15.1 ± 3.4	13.1–16.3
% Preimplantation loss per litter[b]	3.8	3.0	2.3	9.4	0.9–13.6
% Postimplantation loss per litter[c]	4.9	3.3	4.0	6.3	0–11.5
No. of live fetuses per litter[a]	15.4 ± 1.5	15.3 ± 1.3	16.0 ± 1.8	14.2 ± 3.6	12.4–15.5
Sex ratio of live fetuses (male/total)	0.51	0.47	0.48	0.48	0.38–0.59
Body weight of live fetuses (g)[a]					
Male	3.88 ± 0.22	3.87 ± 0.30	3.92 ± 0.19	4.00 ± 0.26	3.56–4.01
Female	3.68 ± 0.19	3.69 ± 0.31	3.70 ± 0.14	3.79 ± 0.29	3.33–3.81

[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]Historical control values were obtained from the studies performed in SNBL during 1996–2004 using Crj: CD (SD) IGS rats.

Table 3: Morphological examinations in fetuses of rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)				
	0 (control)	62.5	250	1000	Historical control values[b]
External examination					
Total no. of fetuses (litters) examined	262 (17)	275 (18)	272 (17)	255 (18)	9178 (652): 48 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–0.8%
Skeletal examination					
Total no. of fetuses (litters) examined	136 (17)	141 (18)	141 (17)	132 (18)	3741 (516): 29 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–1.3%
Total no. of fetuses (litters) with variations	18 (7)	12 (10)	11 (8)	17 (11)	3.6–19.2%
Asymmetry of sternebrae	1	1	0	0	0–2.8%
Dumbbell ossification of thoracic centrum	1	3 (3)	2 (1)	2 (2)	0–5.5%
Splitting of thoracic centrum	0	0	0	1	0–3.0%
Full supernumerary ribs	0	0	1	0	0–4.4%
Short supernumerary ribs	16 (6)	8 (6)	9 (7)	14 (8)	0.3–17.1%
Short 13th ribs	0	0	0	1	0%
Degree of ossification[a]					
No. of sacral and caudal vertebrae	8.0 ± 0.4	8.0 ± 0.5	8.2 ± 0.4	8.1 ± 0.3	7.5–8.4
No. of sternebrae	5.4 ± 0.5	5.5 ± 0.6	5.7 ± 0.3	5.4 ± 0.5	4.7–5.7
Internal examination					
Total no. of fetuses (litters) examined	126 (17)	134 (18)	131 (17)	123 (18)	3459 (510): 30 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–0.8%
Total no. of fetuses (litters) with variations	2 (2)	5 (4)	8 (6)	10 (6)	0–22.4%
Thymic remnants in neck	1	2 (2)	2 (2)	3 (3)	0–10.0%
Dilated renal pelvis	0	0	3 (2)	3 (2)	0–14.2%
Dilated ureter	1	3 (2)	6 (4)	7 (4)	0–14.2%
Convoluted ureter	0	0	0	1	0–3.8%

[a]Values are given as the mean ± SD.
[b]Historical control values were obtained from the studies performed in SNBL during 1996–2004 using Crj: CD (SD) IGS rats.

pelvis, dilated ureter and/or convoluted ureter, were observed in all groups, including the control group. However, no significant differences in the incidences of the total number of fetuses with internal variations and individual internal variation were found between the control and DBHCB-treated groups.

DISCUSSION

The current study was conducted to determine the prenatal developmental toxicity of DBHCB. The data showed that the prenatal oral administration of DBHCB did not produce any adverse effects, including morphological anomalies in fetuses of rats.

DBHCB was given to pregnant rats during the time of implantation to the term of pregnancy, to characterize the effects of DBHCB on embryonic/fetal development. The number of implantations was slightly reduced, and incidence of preimplantation loss was slightly increased in the high-dosage group, a finding associated with the tendency for reduced maternal body weight gain during the administration period, with an increase in maternal body weight gain after completion of the administration period. These differences were probably associated with the variability in litter sizes in the high-dosage group and unrelated to the administration of the test chemical. No significant changes in any maternal parameters were noted, even at 1000 mg/kg. No significant changes in embryonic/fetal survival or growth parameters were found, even at 1000 mg/kg. These findings indicate that DBHCB is not toxic to maternal animals, embryonic/fetal survival, or fetal growth when administered during the time of implantation to the term of pregnancy.

Morphological examinations in the fetuses of exposed mothers revealed no fetuses with external malformations. However, some fetuses with skeletal and/or internal variations were found in all groups. The variations observed in the current study are of the types that occur spontaneously among the control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). A skeletal variation (i.e., full supernumerary ribs) has been described as a warning sign of possible teratogenicity and is known to occur in the presence of perturbation of maternal homeostatis. All other variations, short supernumerary ribs, sternebral variations, and bilobed centra of the vertebral column, are frequent variations, which were considered to be normal findings (Kimmel and Wilson, 1973). Although several types of skeletal variations, including full supernumerary ribs, were found in the control and DBHCB-treated groups, no consistent tendency was noted in the incidence of fetuses with these alterations. No significant differences between the control and DBHCB-treated groups were observed in the incidences of the total number of fetuses with skeletal variations or individual types of skeletal variation. Furthermore, these incidences were within the ranges of the background control data in the laboratory-performed current study. As for the internal variations, there was an increasing trend, according to the increasing doses, in the total number of

fetuses with internal variations and the number of fetuses with dilated renal pelvis or ureter. In the current study, the incidences of fetuses with internal variations, with dilated renal pelvis, and with dilated ureter at 1000 mg/kg were 7.5%, 2.1%, and 5.4%, respectively. In the background control data in the current study, these values were 0–22.4%, 0–14.2%, and 0–14.2% (Table 3). Because the incidences of fetuses with internal variations were within the range of the historical control data, and there were no statistically significant differences between the control and DBHCB-treated groups, these findings were considered unrelated to DBHCB and simply expression of the normal background incidence of such findings. Chahoud et al. (1999) noted that variations are unlikely to adversely affect the survival or health, and this might result from a delay in growth or morphogenesis that has otherwise followed a normal pattern of development. The alterations observed in the current study are not thought to be due to the administration of DBHCB, because they have occurred at a very low incidence and are of types that occur sporadically among control rat fetuses. Consideration of these findings together suggests that the morphological changes in fetuses observed in the current study do not indicate a teratogenic response and that DBHCB possesses no teratogenic potential in rats.

There was no available data for human exposure to this chemical. Actual human exposure to DBHCB may be estimated to be very low, because this chemical was not detected from polyethyleneterephthalate bottles in Brazil (Monteiro et al., 1998) and from polyethylene products in Japan (Kawamura et al., 1997). Consideration of these findings and the results of the current study together suggests that the risk of adverse effects of DBHCB on prenatal development of offspring is very low.

CONCLUSION

The current results showed that the administration of DBHCB to pregnant rats during the time of implantation to the term of pregnancy had no adverse effects on maternal rats and embryonic/fetal development, even at 1000 mg/kg no observed adverse effect levels. Based on these findings, it is concluded that the (NOAELs) of DBHCB for both dams and fetuses were 1000 mg kg^{-1} day^{-1} in rats.

ACKNOWLEDGMENTS

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

REFERENCES

Barnett, J. F., Jr., 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