



Fig. 4. Correlation between pNOAELs and BMDLs from both newborn (closed squares) and young (triangles) studies.

be excluded because they may be extremely sensitive to chemicals due to their impaired capacity for detoxification or excretion. Pregnant women and fetuses are also sensitive subpopulations, but the toxicity to these groups is routinely tested by reproductive and developmental toxicity studies.

Recently, Dourson et al. (2002) summarized considerations on adequacy of uncertainty factors of human variability for children. With human data, Glaubiger et al. (1981) reported that overall differences in sensitivity between children and adults are quite small on the basis of comparing maximum tolerated doses for 17 anticancer drugs. Using animal data analyses, Charnley and Putzrath (2001) demonstrated that younger animals appear to be less susceptible to 47% and more susceptible to 40% of the tested chemicals in carcinogenesis studies. Sheehan and Gaylor (1990) showed newborn mammals to be more sensitive than adults (86% within 10-fold) by comparing LD_{50} ratios for 238 chemicals. Calabrese (2001) also reported that younger animals have a greater sensitivity than older animals in 54% of cases (more than 10-fold in 14%) with LD_{50} ratio analyses for 313 chemicals. All these reports suggest the degree of variation in sensitivity of infants/younger animals as compared to adults for most chemicals may be within 10-fold, so that a 10-fold uncertainty factor may be sufficient to cover the variation (Dourson et al., 2002). However, only Sheehan and Gaylor (1990) targeted newborn rather than young animals and the report was a meeting abstract.

Concerning the methodology for risk assessment with repeated exposure, NOAELs or BMDLs from repeated-dose toxicity studies are starting values to derive risk values such

as acceptable daily intake (ADI) or tolerable daily intake (TDI). These studies might be as short as 28 days or as long as 2 years, but invariably dosing generally starts around 6 weeks of age for rodents. These animals are referred to as “young” in this article rather than adult because their growth is still vigorous. Therefore, toxicity responses of young animals, equivalent to late childhood in humans, may already be covered by the general repeated-dose toxicity studies (see also Table 2 of Dourson et al., 2002 which summarized work by Scheuplein et al., 2002, on this point).

However, only limited data exist for animals from birth to 5 or 6 weeks of age. During these initial few weeks after birth, susceptibility to toxic insult might be expected to be greater than at later periods because organ growth rates are higher. Moreover, metabolism and elimination pathways are not yet mature (see for example the discussion of kinetic comparisons of newborn, infants and children as compared with adults by Rane, 1992 and Renwick, 1998 in Dourson et al., 2002). Although for some chemicals this lack of maturation in metabolism and elimination might serve to protect the newborn, it is clearly very important to clarify newborn sensitivity versus young animal sensitivity. Thus we have designed our newborn rat study protocol (18 day newborn study) to follow the conditions of the 28 day repeated-dose toxicity study (onset of administration at 5–6 weeks old) as closely as possible using 18, mostly phenolic, compounds. In addition to the unique design of the 18 day newborn study, new clarifying terminology has been developed, pNOAELs and pUETLs, in order to more appropriately determine ratios between newborn and young studies.

Our analyses of 18 such pNOAEL ratios or 17 BMDL ratios revealed less or nearly equal sensitivity in newborn animals (less than 2-fold) in 33–53%, clearly greater sensitivity (2–8-fold) in 47–61% and one exceptional case of more than 25-fold sensitivity in the newborn. In the case of 16 pUETL ratios, 31% of chemicals showed less or nearly equal toxicity in newborns (less than 2-fold) and 69% more toxicity (2–8-fold) in newborns. This distribution and the extent of newborn susceptibility in toxicity are in line with the conclusions of several investigators summarized previously by Dourson et al. (2002), but evidence presented here is more direct because of careful design of the comparative studies and comprehensive toxicological analyses and judgments. In addition, two kinetic analyses showing newborns to be more sensitive than adults, with a 3.5 arithmetic average difference in elimination half life (Rane, 1992) or a 4-fold longer average half life (Ginsberg et al., 2002), support relatively similar degrees of average susceptibility as we have found.

Collectively, all of this work suggests that studying the early life stage sensitivity to toxic insult is important. When such studies determine the critical effect, then ADIs or TDIs should be based on their findings. When such studies do not determine the critical effect, then the ADI or TDI is appropriately based on a critical effect found in a different study and the newborn is protected. It is when such studies have not been conducted that uncertainty factors must be invoked to protect the newborn, and other potential sensitive subpopulations, and several investigators have looked at the adequacy of such factors (e.g., Burin and Saunders, 1999; Dourson et al., 2002). Based on our results and those of other investigators, we suggest that an uncertainty factor of 10-fold for human variability and an uncertainty factor of between 3- and 10-fold for database completeness can be considered appropriate for risk assessment unless knowledge of particular toxicity in newborn or infants is present, or if not present is discountable due to other credible information on the chemical.

In conclusion, newborn rats are clearly more susceptible than young animals (at most 8-fold) to two thirds of the present series of 18 chemicals, mostly phenolic substances, and less or nearly equal sensitive to the others for oral repeated exposure. However, it should be noted that there was one exceptional case in which the toxicity appeared only in newborn rats. These repeated oral exposure newborn studies are unique for this limited group of chemicals, and perhaps for other chemicals as well.

Acknowledgments

The authors gratefully acknowledge the financial support of the Office of Chemical Safety, Pharmaceutical and Medical Safety Bureau, Ministry of Health, Labour and Welfare, Japan and also wish to express their deep

appreciation to the five Japanese contract laboratories (Gotemba Laboratory, Bozo Research Center Inc., Panapharm Laboratories Co., Ltd., Research Institute for Animal Science in Biochemistry and Toxicology (Foundation), Mitsubishi Chemical Safety Institute Ltd. and Safety Research Institute for Chemical Compounds Co., Ltd.) for their efforts in performing the actual animal toxicity studies.

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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 LD₅₀ 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

^aValues are given as the mean ± SD.

^bAdjusted 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 sternbrae 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)				Historical control values ^d
	0 (control)	62.5	250	1000	
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

^aValues 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.^dHistorical control values were obtained from the studies performed in SNBL during 1996-2004 using Crlj: CD (SD) IGS rats.

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

	Dose (mg/kg)					Historical control values ^b
	0 (control)	62.5	250	1000		
External examination						
Total no. of fetuses (litters) examined	262 (17)	275 (18)	272 (17)	255 (18)	9178 (652): 48 studies	0-0.8%
Total no. of fetuses (litters) with malformations	0	0	0	0		
Skeletal examination						
Total no. of fetuses (litters) examined	136 (17)	141 (18)	141 (17)	132 (18)	3741 (516): 29 studies	0-1.3%
Total no. of fetuses (litters) with malformations	0	0	0	0		3.6-19.2%
Total no. of fetuses (litters) with variations	18 (7)	12 (10)	11 (8)	17 (11)		0-2.8%
Asymmetry of sternbrae	1	1	0	0		0-5.5%
Dumbbell ossification of thoracic centrum	1	3 (3)	2 (1)	2 (2)		
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 sternbrae	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	0-0.8%
Total no. of fetuses (litters) with malformations	0	0	0	0		0-22.4%
Total no. of fetuses (litters) with variations	2 (2)	5 (4)	8 (6)	10 (6)		0-10.0%
Thymic remnants in neck	1	2 (2)	2 (2)	3 (3)		0-14.2%
Dilated renal pelvis	0	0	3 (2)	3 (2)		0-14.2%
Dilated ureter	1	3 (2)	6 (4)	7 (4)		0-3.8%
Convulsed ureter	0	0	0	1		

^aValues are given as the mean ± SD.

^bHistorical control values were obtained from the studies performed in SNBL during 1996-2004 using Crlj: 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 pre-implantation 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, sternbral 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.

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Prenatal developmental toxicity study of the basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats

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Received 28 December 2005; received in revised form 26 April 2006; accepted 4 May 2006

Available online 16 May 2006

Abstract

Pregnant rats were given 1,3-di-*o*-tolylguanidine (DTG) by gavage at 0, 10, 20 or 40 mg/kg bw/day on days 6–19 of pregnancy and the pregnancy outcome was determined on day 20 of pregnancy. At 40 mg/kg bw/day, deaths were observed in four out of 24 females. The incidences of females showing mydriasis at 20 and 40 mg/kg bw/day and showing decreased locomotor activity at 40 mg/kg bw/day were significantly increased. Alopecia, bradypnea, prone position and tremor were also observed at 40 mg/kg bw/day. The maternal body weight gain at 20 and 40 mg/kg bw/day and food consumption at 40 mg/kg bw/day were significantly reduced. A significantly decreased weight of the gravid uterus, increased incidence of postimplantation loss, decreased number of live fetuses, and lowered weights of fetuses and placentae were found at 40 mg/kg bw/day. The incidences of the total number of fetuses with external malformations at 40 mg/kg bw/day and with skeletal malformations at 20 and 40 mg/kg bw/day were significantly increased. Significantly higher incidences of fetuses with brachydactyly and short tail and defects of caudal vertebrae, phalanges and metacarpals were observed at 40 mg/kg bw/day. Delayed ossification was also noted at 40 mg/kg bw/day. The data indicate that DTG is teratogenic at maternal toxic doses and the NOAELs of DTG for maternal and developmental toxicity are 10 mg/kg bw/day in rats.

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Keywords: Di-*o*-tolylguanidine; Rubber accelerator; Sigma ligand; Prenatal developmental toxicity; Teratogenicity; Malformation; Rat

1. Introduction

1,3-Di-*o*-tolylguanidine (CAS No. 97-39-2; DTG) is produced in the million pound range annually in the USA [1] and used as a basic rubber accelerator [2]. DTG is known to be a selective ligand receptor for the sigma site in the mammalian central nervous system [3]. Many findings have suggested that the sigma site plays a role in movement and posture through its association with brainstem and forebrain motor control circuits [4]. DTG has been reported to cause hypothermia after intraperitoneal injection in mice [5] and subcutaneous or intracerebroventricle injection in rats [6,7]. Intraperitoneal injection of DTG reduced the pain behavior in the acute phase, but increased pain behavior in the tonic phase in the formalin test in mice [8], and produced significant, but short-lived,

increases in the withdrawal latencies in mice [5]. In rats, DTG also caused circling behavior after unilateral intranigral injection [4], decreased locomotor activity after intraperitoneal injection [9,10], increased bladder capacity after intravenous injection in the anaesthetized condition [11], and no change in immobility time in the forced swimming test after intraperitoneal injection [12].

It is generally assumed that the biological effects produced by chemicals should be studied in laboratory animals to investigate possible influences in human health, and the results of animal tests on chemical toxicity are relevant to humans [13]. Toxicological studies on DTG have given little information on acute animal toxicity [14]: intraperitoneal LD50 was 25 mg/kg bw in mice; the oral LD50 was 500 mg/kg bw in rats; the lowest published lethal dose of oral administration was 80 mg/kg bw in rabbits; and the lowest published lethal dose was 120 mg/kg bw after oral administration in mammals, species unspecified. We recently investigated the reproductive and developmental toxicity of DTG, according to the OECD guideline 421 reproduc-

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tion/developmental toxicity screening test in rats given DTG by gavage at 0, 8, 20 or 50 mg/kg bw/day [15], to obtain the preliminary information on the reproductive and developmental effects of DTG, because the testing for reproductive and developmental toxicity has become an important part of the overall toxicology. Males were given DTG for a total of 49 days beginning 14 days before mating, and females were given DTG for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. In this screening study, deaths in both sexes at 50 mg/kg bw/day, lowered body weight gain and food consumption in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day, and neurobehavioral changes such as mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation in both sexes at 20 and 50 mg/kg bw/day were found. Although no effects of DTG were detected on the estrous cyclicity, precoital interval, copulation, fertility and gestation indexes, numbers of corpora lutea and implantations, and gestation length, significant decreases in the number, body weight and viability of offspring and a significant increase in the incidence of fetuses with external malformations were noted at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were frequently observed at the highest dose. The total number of fetuses with external malformations, but not individual malformation, was significantly increased at 50 mg/kg, and the teratogenic effect of DTG was strongly suggested. However, this screening test does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. Only external examination in the newborn rats was performed, and no internal or skeletal examinations were carried out in this screening test. The prenatal developmental toxicity study was therefore conducted to accurately evaluate the developmental toxicity, including the teratogenicity of DTG in rats.

2. Materials and methods

This study was performed in compliance with OECD guideline 414 Prenatal Developmental Toxicity Study [16] and in accordance with the principles for Good Laboratory Practice [17], “Law for the Humane Treatment and Management of Animals” [Law No. 105, October 1, 1973, revised June 15, 2005] and “Standards Relating to the Care and Management, etc. of Experimental Animals” [Notification No. 6, March 27, 1980 of the Prime Minister’s Office].

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 toxic studies, including 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 Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for five 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 sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered tap water ad libitum, and they were maintained in an air-conditioned room at $22 \pm 3^\circ\text{C}$, with a relative humidity of $50 \pm 20\%$, a 12-h light/dark cycle, and ventilation of 10–15 air changes/hour. Virgin female rats were mated overnight with male rats. The day when the sperm in the vaginal smear and/or vaginal plug were detected was

considered to be day 0 of pregnancy. The copulated females were distributed into four groups to equalize the female body weights among groups. The copulated females were housed individually.

2.2. Chemicals and dosing

DTG was obtained from Sumitomo Chemical Co., Ltd. (Tokyo, Japan). DTG, a white powder, is slightly soluble in hot water and alcohol, soluble in chloroform, and very soluble in ether, and its melting point is 179°C , specific gravity is 1.10 and molecular weight is 239.3 [2]. The DTG (Lot no. 34K21) used in this study was 99.5% pure, and it was kept in a dark place at room temperature. The purity and stability of the chemical were verified by analysis before and after the study. Rats were dosed once daily by gastric intubation with DTG at a dose of 0 (control), 10, 20 or 40 mg/kg bw on days 6 through 19 of pregnancy. The dosage levels were determined based on the results of our reproduction/developmental toxicity screening test [15], in which deaths at 50 mg/kg bw/day and neurobehavioral changes and lowered body weight gain and food consumption at 20 and 50 mg/kg bw/day in females, and decreases in the number, body weight and viability of offspring and increased incidence of fetuses with malformations at 50 mg/kg bw/day were found. DTG was suspended in 0.5% (w/v) carboxymethylcellulose–Na solution with 0.1% (w/v) Tween 80. The volume of each dose was adjusted to 5 ml/kg body weight based on daily body weight. The control rats were given only 0.5% (w/v) carboxymethylcellulose–Na solution with 0.1% (w/v) Tween 80. The stability of formulations has been confirmed for up to 8 days. During use, the formulations were maintained under such conditions for less than 7 days, and each formulation was analyzed for concentration of DTG and the results revealed 90.3–99.5% of the intended concentration.

2.3. Observations

All females were observed daily during the pre-administration period and on the day of sacrifice, and twice a day (before and after administration) during the administration period for clinical signs of toxicity. Maternal body weight was recorded on days 0, 3 and 6–20 of pregnancy. Food consumption was recorded on days 0, 3, 6, 9, 12, 15, 18 and 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 was removed from the maternal body and weighed. The numbers of corpora lutea, implantation sites, 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 and alician blue [18] 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 [19], and the thoracic areas were subjected to microdissecting [20] to reveal internal abnormalities.

2.4. Data 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, weight of the gravid uterus, food consumption, numbers of corpora lutea, implantations 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 did not have equivalences, 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 malformations and variations and sex ratio of live fetuses were analyzed using Wilcoxon’s rank sum test. The rates of pregnancy, non-pregnancy and females showing clinical signs of toxicity were analyzed with Fisher’s exact test. The 0.05 level of probability was used as the criterion for significance.

Table 1
Maternal findings in rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
No. of rats	24	24	24	24
No. of pregnant rats	24	24	24	24
Initial body weight	256 ± 13	256 ± 13	256 ± 13	256 ± 13
No. of females showing clinical sign of toxicity				
Death	0	0	0	4
Alopecia	2	2	3	2
Bradypnea	0	0	0	2
Decreased locomotor activity	0	0	1	11**
Mydriasis	0	0	12**	24**
Prone position	0	0	0	3
Salivation	0	0	2	2
Soil of perigenital	0	0	1	4
Tremor	0	0	0	2
Body weight gain during pregnancy (g) ^a				
Days 0–6	40 ± 8	39 ± 8	40 ± 8	39 ± 8
Days 6–15	50 ± 7	49 ± 9	37 ± 11**	23 ± 10**
Days 15–20	77 ± 9	77 ± 9	71 ± 10	47 ± 16**
Days 0–20	167 ± 17	165 ± 21	148 ± 24**	109 ± 21**
Adjusted weight gain ^b	88 ± 15	87 ± 19	77 ± 15	49 ± 17**
Food consumption during pregnancy (g/day) ^a				
Days 0–6	23 ± 2	23 ± 2	23 ± 2	23 ± 2
Days 6–15	26 ± 2	26 ± 2	24 ± 3	20 ± 3**
Days 15–20	28 ± 2	28 ± 3	26 ± 2	22 ± 3**
Days 0–20	25 ± 2	26 ± 2	24 ± 2	21 ± 2**
Weight of gravid uterus (g) ^a	79 ± 10	78 ± 11	72 ± 15	59 ± 10**

^a Values are given as the mean ± S.D.

^b Adjusted weight gain refers to maternal weight gain excluding the gravid uterus.

** Significantly different from the control ($p < 0.01$).

3. Results

Table 1 shows the maternal findings in rats given DTG on days 6–19 of pregnancy. At 40 mg/kg bw/day, death was found on day 8 of pregnancy in two females and on days 7 and 19 of pregnancy in one female each. Statistically significant increases in the incidence of mydriasis occurred at 20 and 40 mg/kg bw/day, and in decreased locomotor activity at 40 mg/kg bw/day. Additional findings that appeared to be treatment related, but not statistically significant were decreased locomotor activity at 20 mg/kg bw/day, salivation and soil of the perigenital area at 20 and 40 mg/kg bw/day, and bradypnea, prone position and tremors at 40 mg/kg bw/day. These signs were observed consistently throughout the dosing period and relatively higher incidences of these signs were noted during the early administration period. Maternal body weight gain was significantly decreased on days 6–15 and 0–20 of pregnancy at 20 mg/kg bw/day, and on days 6–15, 15–20 and 0–20 of pregnancy at 40 mg/kg bw/day. Adjusted weight gain, the net weight gain of maternal rats during pregnancy, and the weight of the gravid uterus were also significantly reduced at 40 mg/kg bw/day. At this dose, food consumption was significantly lowered on days 6–15, 15–20 and 0–20 of pregnancy.

Table 2 presents the reproductive findings in rats given DTG on days 6–19 of pregnancy. No dam with total litter loss was observed in any group. No effects of DTG were

found on the numbers of corpora lutea and implantations, or the incidence of preimplantation loss. At 40 mg/kg bw/day, a significantly increased incidence of postimplantation loss, a decreased number of live fetuses and lowered weights of male and female fetuses and placentae were noted. The sex ratio of live fetuses was significantly reduced in the DTG-treated groups.

The summarized results of external and internal examinations in fetuses of rats given DTG on days 6–19 of pregnancy are shown in Table 3. No fetuses with external malformations were observed in the control group. One fetus with cleft palate was found at 10 mg/kg bw/day. Fetuses with external malformations were found in 13 out of the 328 fetuses (three out of the 24 litters) at 20 mg/kg bw/day and 33 out of the 251 fetuses (11 out of the 20 litters) at 40 mg/kg bw/day, and significantly increased incidence of the total number of fetuses with external malformations was noted at 40 mg/kg bw/day. Incidences of fetuses with brachydactyly and with short tail were increased at 20 and 40 mg/kg bw/day, and significantly increased incidences were found at 40 mg/kg bw/day. As for internal malformations, one fetus each with microphthalmia in the control and 20 mg/kg bw/day groups, one fetus with dilatation of the lateral ventricles in the control group and one fetus with undescended testes in the 40 mg/kg bw/day were observed. Variations in the internal organs were observed in 11–19 fetuses in all groups. However, no significant differences in the incidences of

Table 2
Reproductive findings in rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
No. of litters	24	24	24	20
No. of litters totally resorbed	0	0	0	0
No. of corpora lutea per litter ^a	15.7 ± 2.1	14.8 ± 1.6	14.9 ± 1.9	15.3 ± 1.5
No. of implantations per litter ^a	15.3 ± 1.9	14.7 ± 1.8	14.2 ± 2.7	15.2 ± 1.4
% Preimplantation loss per litter ^b	2.4	0.9	5.6	0.9
% Postimplantation loss per litter ^c	3.5	3.4	4.8	16.4**
No. of live fetuses per litter ^a	14.8 ± 1.9	14.2 ± 2.1	13.7 ± 2.9	12.6 ± 1.9**
Sex ratio of live fetuses (male/female)	0.56	0.49*	0.46*	0.46*
Body weight of live fetuses (g) ^a				
Male	3.64 ± 0.17	3.72 ± 0.18	3.59 ± 0.24	3.19 ± 0.31**
Female	3.42 ± 0.16	3.53 ± 0.25	3.41 ± 0.18	3.03 ± 0.26**
Placental weight (g) ^a	0.47 ± 0.04	0.47 ± 0.03	0.50 ± 0.16	0.40 ± 0.04**

^a Values are given as the mean ± S.D.

^b (No. of preimplantation embryonic loss/no. of corpora lutea) × 100.

^c (No. of resorptions and dead fetuses/no. implantations) × 100.

* Significantly different from the control ($p < 0.05$).

** Significantly different from the control ($p < 0.01$).

fetuses with internal malformations and variations were detected between the control and DTG-treated groups.

The summarized results of skeletal examinations in the fetuses of rats given DTG on days 6–19 of pregnancy are presented in Table 4. Fetuses with skeletal malformations were found in one out of the 184 fetuses (one out of the 24 litters) in the control group, one out of the 176 fetuses (one out of the 24 litters) at 10 mg/kg bw/day, 13 out of the 170 fetuses (six out of the 24 litters) at 20 mg/kg bw/day, and 26 out of the 130 fetuses (12 out of the 20 litters) at 40 mg/kg bw/day. Significantly higher incidences of the total number of fetuses with skeletal malformations were observed at 20 and 40 mg/kg bw/day. Incidences of fetuses with absence, fusion or malposition of the caudal vertebrae and with absence or fusion of phalanges were higher at 20 and 40 mg/kg bw/day, and significantly increased incidences of fetuses with these malformations and fetuses with the absence or

fusion of metacarpals were found at 40 mg/kg bw/day. Although skeletal variations in the vertebral column, ribs and sternbrae were observed in all groups, no significant differences in the incidences of fetuses with skeletal variations were detected between the control and DTG-treated groups. A significantly delayed ossification, as evidenced by the numbers of sacral and caudal vertebrae, sternbrae, and metatarsi, was also noted at 40 mg/kg bw/day.

4. Discussion

In order to obtain further information on the reproductive and developmental toxicity of DTG, the present study was conducted in compliance with OECD guideline 414 Prenatal Developmental Toxicity Study [16]. DTG was given to pregnant rats during the time of implantation to the term of pregnancy to

Table 3
External and internal examinations in fetuses of rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
External examination				
Total no. of fetuses (litters) examined	354 (24)	341 (24)	328 (24)	251 (20)
Total no. of fetuses (litters) with malformations	0	1	13 (3)	33 (11)**
Cleft palate	0	1	0	0
Brachydactyly	0	0	8 (3)	31 (11)**
Short tail	0	0	7 (2)	10 (7)**
Internal examination				
Total no. of fetuses (litters) examined	170 (24)	165 (24)	158 (24)	121 (20)
Total no. of fetuses (litters) with malformations	1	0	1	1
Microphthalmia	1	0	1	0
Dilatation of lateral ventricles	1	0	0	0
Undescended testes	0	0	0	1
Total no. of fetuses (litters) with variations	16 (10)	11 (9)	13 (7)	19 (12)
Thymic remnants in neck	13 (10)	8 (7)	12 (7)	17 (11)
Dilated renal pelvis	2 (2)	2 (2)	0	0
Left umbilical artery	1	1	1	2 (2)

** Significantly different from the control ($p < 0.01$).

Table 4
Skeletal examinations in fetuses of rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
Total no. of fetuses (litters) examined	184 (24)	176 (24)	170 (24)	130 (20)
Total no. of fetuses (litters) with malformations	1	1	13 (6)*	26 (12)**
Split cartilage of thoracic centrum	0	0	1	1
Fused cartilage of cervical vertebral arches	0	1	1	1
Fused cartilage of ribs	1	0	0	0
Absence, fusion or malposition of caudal vertebrae	0	0	8 (3)	10 (8)**
Absence or fusion of phalanges	0	0	5 (3)	18 (9)**
Fusion of metacarpal/metatarsal and phalanx	0	0	0	2 (2)
Absence or fusion of metacarpals	0	0	0	4 (4)*
Shortening of tibia and fibula	0	0	0	1
Total no. of fetuses (litters) with variations	10 (7)	16 (9)	16 (11)	12 (8)
Bipartite ossification of thoracic centrum	0	2 (1)	1	0
Dumbbell ossification of thoracic centrum	0	1	0	0
Unossified thoracic centrum	1	1	0	1
Variation of number of lumbar vertebrae	1	0	0	2 (1)
Wavy ribs	0	1	1	0
Short supernumerary rib	9 (6)	12 (7)	14 (10)	4 (4)
Short 13th rib	0	0	0	2 (2)
Sacralization of lumbar vertebra	0	0	0	2 (1)
Bipartite ossification of sternebra	0	0	1	1
Asymmetry of sternebra	0	0	0	1
Degree of ossification ^a				
No. of sacral and caudal vertebrae	7.3 ± 0.5	7.5 ± 0.5	7.5 ± 0.5	7.0 ± 0.6*
No. of sternebrae	4.6 ± 0.4	4.8 ± 0.5	4.6 ± 0.4	4.2 ± 0.4*
No. of metatarsals	8.0 ± 0.0	7.9 ± 0.3	7.8 ± 0.4	6.7 ± 1.4*

^a Values are given as the mean ± S.D.

* Significantly different from the control ($p < 0.05$).

** Significantly different from the control ($p < 0.01$).

characterize the effects of DTG on embryonic/fetal development. The findings of the present study confirmed the results of a previous screening study and extended the understanding of the reproductive and developmental toxicity of DTG. The present data showed that the prenatal oral administration of DTG produced maternal toxicity, as evidenced by deaths, neurobehavioral changes, decreased body weight gain and reduced food consumption, and developmental toxicity, as evidenced by a high incidence of postimplantation loss, a decreased number of live fetuses and lower weight of fetuses, and teratogenicity, as evidenced by a higher incidence of fetuses with external and skeletal malformations.

DTG is a specific sigma receptor ligand [3] and sigma receptor ligands can modulate neurotransmissions, including the noradrenergic, glutamatergic and dopaminergic system [10,21,22]. The systemic injection of DTG has been reported to cause neurobehavioral changes in rats [4,6,7,9,22]. The present study shows that the oral administration of DTG also induced neurobehavioral changes at 20 and 40 mg/kg bw/day in pregnant rats. Lowered body weight gain at 20 and 40 mg/kg bw/day and food consumption at 40 mg/kg bw/day were also observed in pregnant rats. These findings indicate that DTG is maternally toxic at 20 mg/kg bw/day and higher.

The sex ratio (males/females) was significantly lowered in all DTG-treated groups. The values for sex ratio were 0.429–0.521 in the background control data for the last 6 years in the labo-

ratory performed present study. Statistically significant changes in the sex ratio observed in the present study were considered to be unrelated to the administration of DTG, because the values for sex ratio in the DTG-treated groups were within the range of the historical control data, no increased embryonic/fetal deaths were detected at 10 and 20 mg/kg bw/day and the control value for the sex ratio was very high in the present study. A decreased number of live fetuses, increased incidence of postimplantation loss, and reduced weights of fetuses and placentae were detected at 40 mg/kg bw/day. A decreased number of live fetuses and increased incidence of postimplantation loss indicate embryonic/fetal lethality, and reduced weights of fetuses and placentae indicate intrauterine growth retardation. These findings indicate that DTG is toxic to embryonic/fetal survival or fetal growth at 40 mg/kg bw/day when administered during the time of implantation to the term of pregnancy.

In our previous reproductive and developmental screening test [15], the total number of fetuses with external malformations, but not individual malformation, was significantly increased at 50 mg/kg. At this dose, oligodactyly and tail anomalies were frequently observed, and the teratogenic effect of DTG was strongly suggested. No malformed fetuses were found at 20 mg/kg bw/day in our previous study. In the present study, morphological examinations in the fetuses of exposed mothers revealed increased incidence of fetuses with external and skeletal malformations at 20 and 40 mg/kg bw/day.