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 is anxious 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 present 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 one 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-hour light/dark cycle, and ventilation with 15 air charges/hour. Virgin female rats were mated overnight with male rats. The day when the sperm and/or vaginal plug was considered 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/day and higher, but not in females even at 1000 mg/kg/day, 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 pre-administration period and twice a day (before administration and one to two hours 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, 1995) 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.

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 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 present 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 present 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 present 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 present 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 present 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 present 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 present study do not indicate a teratogenic response and that DBCHB possesses no teratogenic potential in rats.

There was no available data for human exposure to this chemical. Actual human exposure to DBCHB may be estimated to be very low, because this chemical was not detected from polyethylenterephthalate 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 present 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, a limited dose. Based on these findings, it is concluded that the NOAELs of DBHCB for both dams and fetuses were 1000 mg/kg/day in rats.

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**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.

**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) x 100.
[c] (No. of resorptions and dead fetuses/no. implantations) x 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 13$^{th}$ 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.