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# Effects of 2-Chlorodibenzofuran on Fetal Development in Mice

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2-Chlorodibenzofuran (2-MCDF), a monochlorinated derivative of dibenzofuran, has been detected as a contaminant in chlorinated tap water in Japan. Shiraishi et al. (1985) analyzed polynuclear aromatic hydrocarbons in Tsukuba tap water and detected 0.04 - 1.4 ng/L of 2-MCDF. This means that unintended human exposure to 2-MCDF has occurred, and therefore safety evaluation of 2-MCDF is needed. Toxicological information on 2-MCDF is limited and available only on mutagenicity and metabolic fate. In the Ames test, 2-MCDF showed weak mutagenic activity on *Salmonella typhimurium* strain TA 98 above 0.4 μmol/plate, but not on strain TA 100 even at 10 μmol/plate, and this activity was diminished by the addition of S9 mix (Matsumoto et al. 1988). After intravenous or oral administration to rats, 2-MCDF is rapidly metabolized and excreted in bile and urine (Tohyama et al. 1992). Major metabolites in the rat bile fluid were 2- and/or 7-hydroxylated forms of 2-MCDF (Hirano et al. 1991).

For developmental toxicity, we previously examined the embryotoxicity of 2-MCDF by using post-implantation rat embryo culture (Usami et al. 1993). When day 9 embryos were continuously exposed throughout 48 hr culture period either in the presence or in the absence of a metabolic activation system, 2-MCDF at 1 m*M* was embryotoxic and caused morphological abnormalities of the embryos. However, dosing of up to 1000 mg/kg/day of 2-MCDF to pregnant rats during days 9 to 11 of gestation, corresponding to the culture period, had no effects on the embryo-fetal growth. It was tentatively concluded from these results that 2-MCDF had weak-inactive teratogenicity in rats. In the present study, we further examined fetal effects of 2-MCDF by a teratogenicity test in which the dosing period covers the major organogenic period. We used mice, since they have been used for the evaluation of teratogenicity of polychlorinated congeners of 2-MCDF, because of their higher susceptibility than that of rats (Birnbaum et al. 1987, Couture et al. 1989).

## MATERIALS AND METHODS

Specific-pathogen-free ICR mice (Crj: CD-1) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Five females (8 wk old) or two males (9 wk old)were housed in a polypropylene cage, and were given laboratory chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. The animal room was maintained at a temperature of 24±1°C, relative humidity of 55±5%, ventilation of 14-18 times/hr and a 12-hr light/dark cycle (light on 0600 to 1800).

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For mating, five females were housed with two males overnight. Females with a vaginal plug the following morning were used as pregnant mice at day 0 of gestation. One hundred and twenty pregnant mice in total were obtained and were divided into four groups on the basis of their body weight at day 0 of gestation so that each group consisted of 30 pregnant mice. Data were collected from only those pregnant mice for which pregnancy was verified at sacrifice on day 18 of gestation.

2-MCDF (CAS 51230-49-0) was purchased from and synthesized by Sorl Laboratory (Mie, Japan) with analysis data on its purity of 99.3%. 2-MCDF was suspended in sesame oil and given to the pregnant mice by gavage via stomach tube once a day from days 6 to 15 of gestation. Three 2-MCDF dose groups (125, 250 and 500 mg/kg/day) and a control group were employed. The dosing volume was 5 ml/kg/day on the basis of the body weight at the beginning of the dosing period. The animals in the control group received sesame oil in the same way.

Clinical observation of the pregnant mice was made twice a day before and after the daily dosing during the dosing period, or at least once a day the other periods. The body weight and food consumption of pregnant mice were measured on designated days of gestation. The pregnant mice were sacrificed on day 18 of gestation under deep ether anesthesia. The gravid uterine horns, liver, thymus and spleen were removed and weighed. The number of corpora lutea, dead implants and live fetuses were counted. The live fetuses were sexed, weighed and examined for gross malformation. Half of the live fetuses in each litter were fixed in Bouin's solution, and examined for malformations of the head and abdominal regions by Wilson's method (Wilson 1965) and the thoracic region by the micro-dissection method (Nishimura 1974). The other half were examined for malformations and variations of the skeletal system after KOH clearing and staining with alizarin red S (Dawson 1926).

A litter was used as a sample unit, and the statistical significance of the differences between the control and the 2-MCDF dose groups was examined at 1% and 5% probability levels. The Fisher's exact test was used for categorical data. The Kruskal-Wallis H test followed by the Scheffé test was used for non-parametric data and for those parametric data without homogeneous variances among the groups as determined by the Bartlett test. One-way analysis of variance followed by the Sheffé test was used for parametric data with homogeneous variances among the groups.

## RESULTS AND DISCUSSION

Table 1 shows the effects of 2-MCDF on the pregnant mice. There were slight but significant increases in liver weight of the pregnant mice at 125 and 500 mg/kg/day. In relative liver weight based on the corrected body weight, there was a significant increase at every 2-MCDF dose with a dose-response relationship. These increases in liver weight of the pregnant mice seem to be due to the microsomal-inducing activity of 2-MCDF. This is because 2-MCDF is a microsomal-inducing agent of the 3-methylcholanthrene type (Miura and Takahashi 1990), and because agents of this type can cause a slight increase in liver weight (Sipes and Gandolfi 1986). Although the mutagenicity of 2-MCDF is metabolically inactivated (Matsumoto et al. 1988), it is unknown if the induced microsomal activity reduces maternal or fetal toxicity of 2-MCDF.

Thymus and spleen weights of the pregnant mice were not significantly different, although thymus weight tended to decline in a dose-dependent manner. This is in

Table 1. Body weight and organ weight of pregnant mice treated with 2-chlorodibenzofuran (2-MCDF)

| | Dose (mg/kg/day) | | | |
|---|---|---|---|---|
| | 0 (control) | 125 | 250 | 500 |
| No. of pregnant mice | 21 | 20 | 23 | 22 |
| Final body weight (g)[a] | 56.5 ± 5.12 | 59.0 ± 4.17 | 55.7 ± 5.95 | 56.5 ±5.66 |
| Absolute organ weight[a] | | | | |
| Liver weight (g) | 2.67 ± 0.33 | 3.02 ± 0.31* | 2.96 ± 0.27 | 3.35 ± 0.46** |
| Thymus weight (mg) | 18.6 ± 6.15 | 18.2 ± 6.61 | 16.0 ± 5.24 | 15.7 ± 4.94 |
| Spleen weight (mg) | 124 ± 34.3 | 134 ± 27.3 | 125 ± 34.3 | 135 ± 25.0 |
| Gravid uterine weight (g) | 21.0 ± 3.83 | 22.3 ± 3.65 | 20.3 ± 4.84 | 20.3 ± 4.95 |
| Corrected body weight (g)[ab] | 35.5 ± 2.95 | 36.7 ± 3.55 | 35.5 ± 2.15 | 36.2 ± 1.97 |
| Relative organ weight[ac] | | | | |
| Liver weight (g/100gCBW) | 7.52 ± 0.51 | 8.25 ± 0.60* | 8.37 ± 0.74** | 9.25 ± 0.97** |
| Thymus weight (mg/100gCBW) | 52.4 ± 16.2 | 49.0 ± 14.9 | 45.2 ± 14.8 | 43.6 ± 14.4 |
| Spleen weight (mg/100gCBW) | 349 ± 86.8 | 363 ± 63.1 | 353 ± 99.7 | 372 ± 64.8 |

a, Mean ± S.D. is shown. b, Corrected body weight is final body weight minus gravid uterine weight. c, Calculated from the corrected body weight (CBW). *, Significantly different from the control ($p<0.05$). **, Significantly different from the control ($p<0.01$).

accordance with a structure-activity relationship that those chlorinated dibenzofurans with fewer than four lateral chlorine substituents generally cause no thymic atrophy (Bandiera et al. 1984). No clinical signs were observed, and there were no significant differences in body weight and food consumption of the pregnant mice.

Table 2 shows fetal growth in the pregnant mice treated with 2-MCDF. There were no significant differences in the number of live fetuses, fetal weight and mortality of implants. Some gross malformations, such as exencephaly, open eyelid, cleft palate, club foot and kinky tail, were observed in up to six fetuses in each group, but there were no significant differences in their incidences. On visceral examination, dilated lateral ventricle, hypoplastic thymus, intraperitoneal hemorrhage and renal dysplasia were observed in only one or two fetuses at 250 and 500 mg/kg/day, but there were no significant differences in their incidences. Left-sided umbilical artery was observed in 8 to 17 fetuses in every group, but their incidences were insignificant. Skeletal malformations were observed only at 250 mg/kg/day, and their incidences were insignificant. The observed skeletal malformations were wavy or fused ribs, fused or split vertebrae and 25- or 27-presacral vertebrae.

Skeletal variations observed are shown in Table 3. The total incidences of variations were not significantly increased, but the incidence of the hypoplastic supraoccipital bone was significantly higher, as much as 5.2-fold, at 500 mg/kg/day than in the control group. Most of the hypoplastic supraoccipital bones were narrowed or split at the middle as shown in Figure 1. The increased incidence of this skeletal variation was the sole effect of 2-MCDF on the fetuses. The incidences of lumbar rib were not significantly different although they tended to increase in a dose-dependent manner. The numbers of sacro-caudal vertebrae, an ossification index, were not significantly different either.

These results indicate that 2-MCDF has no dysmorphogenetic effects on mouse fetuses even at maternotoxic levels. Increased liver weight was the maternal toxicity observed at every dose employed. In the fetuses, on the contrary, no toxic effects were observed, other than the increased incidence of a skeletal variation at the high dose. In cultured rat embryos, 2-MCDF caused morphological abnormalities, mainly unclosed posterior neuropore and coelomic hemorrhage at 1 m*M* either in the presence or in the absence of a metabolic activation system composed of rat liver S9 and cofactors (Usami et al. 1993). Pathological consideration of these *in vitro* morphological abnormalities let us suspect teratogenic activity causing spina bifida and intestinal hemorrhage in the fetuses respectively, but no such activities were observed in the present study. A possible explanation to this discrepancy is that the *in vivo* doses were not sufficiently high to cause the malformations even at maternotoxic levels. Although 500 mg/kg, the highest dose employed could be simply calculated at about 2.5 m*M*, 2-MCDF tends to accumulate in the adipose tissue (Tohyama et al. 1992), and accordingly its concentration in the embryos might be lower than 1 m*M*. Another explanation is that 2-MCDF was detoxified by an *in vivo* metabolic system other than liver S-9 fraction and/or was excreted rapidly. An increased incidence of lumbar rib, a possible indicator of chemical teratogenicity (Kimmel and Wilson 1973, Yasuda and Maeda 1972), was not observed either. It is therefore considered that 2-MCDF is nonteratogenic in mice.

Table 2. Fetal growth in pregnant mice treated with 2-chlorodibenzofuran (2-MCDF)

| | Dose (mg/kg/day) | | | |
|---|---|---|---|---|
| | 0 (control) | 125 | 250 | 500 |
| No. of litters | 21 | 20 | 23 | 22 |
| No. of corpora lutea[a] | 14.5 ± 1.9 | 14.9 ± 2.0 | 13.5 ± 2.5 | 14.3 ± 1.8 |
| No. of implants[a] | 13.4 ± 2.4 | 14.2 ± 2.0 | 12.8 ± 2.8 | 12.7 ± 3.5 |
| No. of live fetuses[a] | 12.4 ± 2.7 | 13.2 ± 2.3 | 12.1 ± 3.2 | 12.1 ± 3.4 |
| Sex ratio (male/female) | 0.93 | 1.05 | 1.00 | 1.26 |
| Fetal weight (g)[a] | | | | |
| Male | 1.39 ± 0.07 | 1.41 ± 0.09 | 1.38 ± 0.08 | 1.38 ± 0.15 |
| Female | 1.34 ± 0.09 | 1.35 ± 0.08 | 1.33 ± 0.08 | 1.32 ± 0.12 |
| Mortality of implants (%)[a] | 7.3 ± 12.2 | 7.2 ± 7.4 | 6.9 ± 8.7 | 4.3 ± 7.2 |
| No. of fetuses with malformation[b] | | | | |
| Gross malformation | 7 (2.56%) | 4 (1.33%) | 8 (2.88%) | 8 (2.77%) |
| Visceral malformation | 13 (10.6%) | 8 (6.48%) | 20 (13.8%) | 16 (10.6%) |
| Skeletal malformation | 0 (0.00%) | 0 (0.00%) | 3 (2.54%) | 0 (0.00%) |

a, Mean ± S.D. is shown. b, Total number and mean incidence are shown.

Table 3. Skeletal variations in the fetuses from pregnant mice treated with 2-chlorodibenzofuran (2-MCDF)

| | Dose (mg/kg/day) | | | |
|---|---|---|---|---|
| | 0 (control) | 125 | 250 | 500 |
| No. of litters examined | 21 | 20 | 23 | 22 |
| No. of fetuses examined | 133 | 137 | 142 | 137 |
| No. of fetuses with variation[a] | 81 (59.9%) | 75 (55.3%) | 76 (53.8%) | 100 (71.4%) |
| Hypoplastic supraoccipital | 2 (1.63%) | 2 (1.08%) | 3 (2.69%) | 12 (8.54%)* |
| Cervical rib | 32 (24.1%) | 24 (18.0%) | 20 (13.8%) | 35 (25.1%) |
| Deformed sternebrae | 45 (31.9%) | 40 (29.0%) | 40 (28.7%) | 57 (39.5%) |
| Lumbar rib[b] | 25 (18.8%) | 25 (20.0%) | 35 (27.1%) | 45 (33.3%) |
| Extra | 14 (11.4%) | 11 (9.01%) | 19 (16.6%) | 23 (17.1%) |
| Rudimentary | 18 (12.7%) | 16 (12.3%) | 23 (15.8%) | 27 (19.8%) |
| Others | 10 (7.91%) | 7 (4.76%) | 7 (5.19%) | 4 (4.58%) |
| No. of sacro-caudal vertebrae[c] | 12.0 ± 1.00 | 12.8 ± 1.04 | 12.3 ± 1.14 | 11.9 ± 1.41 |

a, Total number and mean incidence are shown. b, Those lumbar ribs longer than half the length of the 13th rib are classified as extra and shorter ones are classified as rudimentary. c, Mean ± S.D. is shown.
*, Significantly different from the control ($p<0.05$).

Fig. 1. Dorsal view of the normal (left) and hypoplastic (right) supraoccipital bones in fetal mice. The hypoplastic one is split at the middle.

From the fetal and maternal effects described above, the acute no observable effect level (NOEL) for mouse fetuses was estimated to be 250 mg/kg/day, and for pregnant mice, lower than 125 mg/kg/day. On the basis of this fetal NOEL, the allowable daily intake (ADI) for human is calculated at 2.5 mg/day, assuming that body weight of a mother is 50 kg and the safety factor is as high as 5,000 (Hogan and Hoel 1989). This ADI value is corresponding to drinking $1.79 \times 10^3$ L/day of water containing 2-MCDF of the maximum concentration detected (1.4 ng/L). It is therefore considered that there appears no risk of 2-MCDF to human fetuses at the concentrations in chlorinated tap water provided that mice are an accurate model of humans for this chemical.

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