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Original Article

Maternal Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin and the Body Burden in Offspring of Long-Evans Rats

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Abstract

Objectives: *In utero* and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) results in a wide variety of developmental effects in pups at doses much lower than those causing overt toxicity in adult animals. We investigated the relationship between tissue concentrations of TCDD in dams and fetuses and developmental effects on pups.

Materials and Methods: Pregnant Long-Evans rats were given TCDD at a single oral dose of 12.5, 50, 200, or 800 ng of TCDD or [^{3}H]-TCDD/kg bw on gestation day (GD) 15. Dams were sacrificed on GD16 and GD21, and the tissue concentrations of TCDD were measured in dams and fetuses. Pups were sacrificed on postnatal day (PND) 49 and PND63 for males and PND70 for females, and the reproductive effects and tissue concentrations of TCDD were determined.

Results: The sex ratio (male/female) on GD21 was significantly reduced at 50 ng TCDD/kg and at 12.5 and 50 ng TCDD/kg at birth, but not at other doses. Delayed puberty was observed in males at 200 ng TCDD/kg and in males and females at 800 ng TCDD/kg. Anogenital distance, testis weight, epididymal sperm count, sperm motility, and ejaculated sperm count were not affected. Estrous cyclicity was not different from that of the control in any treatment group. A dose-dependent decrease in weight of seminal vesicle and prostate on PND49 was observed. Prostate weight was significantly decreased at 800 ng TCDD/kg. At this dose, maternal body burden and TCDD concentration in fetuses were 290 pg TCDD/g and 52 pg TCDD/g on GD16, respectively.

Reduced prostate weight is a sensitive and commonly observed endpoint so that the body burdens of dams and fetuses at the LOAEL of this endpoint could be served as the basis for establishing TDI for dioxins.

Key words: TCDD, *in utero* and lactational exposure, male reproduction, body burden, fetus

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent and prototypical congener of dioxins and related compounds found ubiquitously in the environment and identified as compounds potentially hazardous to human health. Endocrine disrupting effects of dioxins have gained much attention since endocrine disrupting effects by TCDD are thought to be responsible for reproductive and developmental disorders in man and laboratory animals (1–4).

In 1998, World Health Organization (WHO) consultation revised the tolerable daily intake (TDI) as a range of 1–4 pg TEQ/kg bw, based on the developmental, reproductive, and endocrine effects of maternal exposure to TCDD on rats and monkeys (5). The adoption of these data took over the chronic toxicity data, such as liver toxicity or carcinogenicity reported in long-term rat studies (6) that had been used for setting up the TDI value until 1998. At the consultation of WHO, the body burden concept was introduced as described below, and these developmental, reproductive and endocrine effects turned out to be the most sensitive adverse effects so far reported. Among them, the decreased sperm count in offspring of Holtzman rats

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were found to be caused at the lowest dose, 64 ng/kg bw (7). Although there have been several studies using an identical experimental protocol, the effects on the reproductive system of male offspring have not been always consistent with one another (7–11). The issue of consideration is whether TDI be derived from either the most sensitive endpoint reported in only one study or the sensitive endpoints identified commonly in several studies.

The WHO consultation in 1998 introduced the concept of "body burden", as a dose metric, that could replace the commonly-used administered dose when extrapolating animal data to humans. The body burden of TCDD in dams and fetuses/pups following a single oral dose of TCDD on GD15 was often estimated from the administered dose, but in some cases particular endpoints were correlated to the TCDD body burden that was estimated from the TCDD concentrations actually determined in a separate study at the same laboratory (12). As mentioned above, the effects of TCDD on rat fetuses/pups varied from study to study and from strain to strain. It would be preferable to conduct a dose-response study and a distribution study in the same set of experiments to relate the body burden to the endpoints.

In the present study, the effects of TCDD on fetuses/pups and the distribution of TCDD in dams and fetuses/pups following *in utero* and lactational TCDD exposure in Long Evans (LE) rats were investigated in the same set of experiments to explore the common sensitive endpoints in rat offspring and the body burdens at the LOAELs of these endpoints.

Materials and Methods

Animals

Male and female LE rats were purchased from Charles River Laboratories (Wilmington, MA, USA), bred and maintained in a controlled environment at Panapharm Laboratories (Uto, Japan) with temperature at 24±2°C, humidity at 55±10%, and a 12/12-h light/dark cycle (lighted from 0700 to 1900h). They were given food and water *ad libitum*. Female rats (12-week-old) were mated 1:1 with males overnight, and females that had sperm in a vaginal smear or a vaginal plug in the following morning (0900h) were designated as Day 0 of gestation. Dams were housed individually in clear plastic cages with wood chips as bedding. All animal experiments were performed according to the guidelines for animal experiments at the National Institute for Environmental Studies.

Chemicals

TCDD (purity, 99.0%; 50 μg/ml nonane solution) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). 2,3,7,8-Tetrachloro [1,6-^{3}H] dibenzo-*p*-dioxin (^{3}H-TCDD) (purity, >97.0%; specific activity 33.4 Ci/mmol) was obtained from Nemoto & Co., Ltd. (Tokyo, Japan). Corn oil used for dissolving TCDD or vehicle was obtained from Nacalai Tesque (Kyoto, Japan).

Experimental design and treatment

For the dose-response study, twenty-two dams were assigned to each group. On GD15, pregnant rats were given TCDD at a single oral dose of 12.5, 50, 200 or 800 ng/kg (5.0 ml/kg) or an equivalent volume of vehicle. As GD15 was reported to be a critical window for male developmental reproductive toxicity (7, 10, 13), a similar experimental protocol as Mably's was used (7). Five dams from each group were euthanized by diethyether anesthesia on GD16 and GD21. Twelve dams per group were allowed to deliver and necropsied on postnatal day (PND) 22. Pups were randomly culled to adjust the litter size (4 males and 4 females, if possible) on PND 4 and numbered for identification. On PND49 and PND63, male pups numbered 1 and 2 in each litter were sacrificed. Female pups numbered 1 in each litter were sacrificed on PND70.

For the distribution study, fifteen dams were assigned to each group. On GD15, dams were given TCDD at a single oral dose of 12.5, 50, 200 or 800 ng/kg (5.0 ml/kg) or an equivalent volume of vehicle. The specific activity of the dosing solution was 0.047, 0.189, 0.757, or 0.757 MBq/kg, respectively. Five dams from each group were sacrificed on GD16 and GD21. Five dams per group were allowed to deliver; one male pup from each litter was sacrificed on PND49 and PND63, and one female pup from each litter was sacrificed on PND70.

The general health condition of the animals was examined everyday. Body weight, as well as food and water consumption, was measured periodically. At necropsy, dams (GD16, GD21 and PND22) were anesthetized with diethylether and offspring were anesthetized with sodium pentobarbital. Blood was collected from the inferior vena cava and euthanized by exsanguinations. Organs were removed, dissected free of connective tissues and weighed. In the dose-response study, organs were removed and fixed in 10% buffered formalin, except for the testes and epididymides, which were fixed in Bouin's fixative for histopathological examination.

Dose-response study

Dams

The ovaries and uterus were removed, and the numbers of copora lutea and implantation sites were recorded. Brain, heart, lung, thymus, liver, spleen, kidneys, and adrenals were removed.

Fetus

On GD16 and GD21, the number of early and late resorptions and the number of live and dead fetuses were recorded. Live fetuses were weighed individually and examined for sex and external malformations. On GD21, the brain, liver, thymus, and genital organs from a male and a female fetus from each litter were removed.

Pups

The litter size, the number of live and dead pups, and the sex of each pup were recorded after delivery. The weight of each pup was measured. External abnormalities were noted. Maternal/pup viability and growth were monitored throughout the study. Weaning of each litter was conducted on PND21. All pups were examined for eye opening on PND13. Age and body weight at preputial separation (the separation of the foreskin of the penis from the glans penis) were determined from PND42 and thereafter. Age and body weight at the time of vaginal

opening were assessed from PND32 and thereafter. Estrus cyclicity was assessed from PND56 to PND69.

On PND49 and PND63 (male) or PND70 (female: because females were examined estrus cyclicity from PND56 to PND69), 12 rats per dose group (1 pup per each litter) were necropsied to collect blood, brain, pituitary, thymus, heart, spleen, pancreas, thyroid, adrenal glands, kidneys, liver, prostate (male), seminal vesicle (male), testes (male), epididymis (male), ovaries (female), and uterus (female).

At necropsies on PND49 and PND63, anogenital distance (the length between the base of the genital tubercle and the anterior edge of the anus) was measured with a caliper. The right cauda epididymis was excised, weighed and gently chopped in a petri dish with prewarmed (37°C) 5.0 ml of Hank's Balanced Salt Solution (HBSS) containing 0.5% BSA. The petri dish was swirled to disperse the sperm. A small amount of this original sperm suspension was dropped into a HBSS on a prewarmed slide (37°C). The sample was coverslipped and examined for percentage of motile sperm. Epididymal sperm count was performed as follows. The original sperm suspension was filtered with gauze and diluted to 10.0 ml with PBS containing 0.5% formalin. This sperm suspension was thoroughly mixed and diluted to obtain a sperm count of 30 to 100 in a hematocytemeter. Three aliquots of this suspension were counted per sample. The data were reported as the number of cauda epididymal sperm per milligram of tissue.

Ejaculated sperm counts

At 12 or 13 weeks of age, females were mated 1:1 with males of the same dose group overnight. The females which had a vaginal plug the following morning (0900h) were sacrificed just after inspection; their uterus and vagina were removed and flushed using HBSS. The sperm in the flushing buffer was counted with a hematocytometer as mentioned above.

Histopathology

The fixed organs of dams, fetuses, and offspring from the control group and the highest-dose group were embedded in paraffin. Deparaffinized sections (5 μm-thick) were stained with hematoxylin and eosin and examined for histopathological lesions.

Distribution study

Dams were necropsied on GD16 and GD21. Blood, maternal organs and tissues (brain, pituitary, thyroid, thymus, heart, lungs, liver, kidneys, adrenal glands, pancreas, uterus, ovaries, muscle, fat, stomach, and small and large intestines) and fetuses were removed and weighed. On GD16, two fetuses from each litter were randomly selected to determine the radioactivity in each fetus. On GD21, two fetuses from each litter were randomly selected, blood was removed via the carotid vein, and the brain, heart, lung, liver, kidneys, and spleen were removed to determine the radioactivity.

Male offspring (one pup per each litter) and female offspring (one pup per each litter) were necropsied on PND49 and PND63, and on PND70, respectively. Blood, brain, pituitary, thyroid, thymus, heart, lungs, liver, kidneys, adrenal glands, spleen, pancreas, prostate (male), testes (male), seminal vesicle (male), and uterus (female), stomach, small and large intestines were removed and weighed. As for stomach, small and large intestines, their contents were removed and washed by distilled water.

Determination of radioactivity

An aliquot of serum was dissolved in 0.1 ml of Soluene, and 6 ml of scintillator was added. Blood and tissues (including fetuses of GD16) were subject to decoloration by using a 30% hydrogen peroxide solution and dissolved in 0.5–1.5 ml Soluene with an addition of 15 ml of scintillator. In the wet method, intact samples were processed as mentioned above. In the dry method, samples were dried, dehydrated and then processed as mentioned above. Radioactivity was determined using a liquid scintillation counter (Tri Carb 2500TR or 2700TR, Packard, Downers Grove, IL, USA) for 5.0 min.

Non-pregnant female rats

Non-pregnant female LE rats (5 rats per group) were given ^{3}H-TCDD at a single oral dose of 12.5, 50, 200, or 800 ng/kg and necropsied at 1, 6, and 27 days after dosing. Sample collection and determination of radioactivity were conducted in the same way as for the dams.

Data analysis

Statistical analysis was performed using SAS statistical software (Release 6.12) (SAS Institute Japan, Tokyo, Japan). Body weight, organ weight and reproductive indices of dams were analyzed by Dunnett's (homogeneity of variance) or Steel's (heterogeneity of variance) multi-comparison test according to the homogeneity of variance based on the outcome of Bartlett's test for equality of variance. Sperm counts were also processed as mentioned above. Mann-Whitney U-test for histopathological data and χ^2-test for gestational index [(number of females with live newborn/number of pregnant females)×100] and sex ratio were performed. Fischer's exact test for necropsy data and Wilcoxon's Rank-Sum test for fertility indices, birth rate, dead birth rate, ratio of fetuses or pups with external abnormality, ratio of pups alive at PND4, cumulative frequencies of eye opening, preputial separation, vaginal opening and mobile sperm rate were performed. Significance was set at $p<0.05$.

Results

Maternal and preweanling pup data

TCDD administration at doses of 12.5 to 800 ng/kg did not cause any significant changes in the general health condition, body weight, food and water consumption, relative organ weight, and histopathology of dams (data not shown). At necropsy on GD16 and 21, *in utero* exposure to TCDD did not cause any significant difference from the control group in the number of corpola lutea, number of implantation sites, fetal mortality, resorption rate, or rates of dead and live fetuses in treatment groups. On GD21, the sex ratio at the 50 ng/kg group was significantly less than that of the control group (Table 1).

As summarized in Table 2, sex ratios of live newborns exposed *in utero* to TCDD at a dose of 12.5 ng/kg and 50 ng/kg were significantly lower than vehicle-treated control. The

Table 1 Reproductive outcomes of dams treated orally with 2,3,7,8-TCDD on day 15 of gestation (necropsied on day 21 of gestation)

Group and dose	Control	12.5 ng/kg	50 ng/kg	200 ng/kg	800 ng/kg
No. of dams	5	5	5	4	5
No. of corpora lutea[a)]	76 (15.2±3.03)	94 (18.8±2.39)	79 (15.8±2.86)	61 (15.3±1.50)	81 (16.2±1.48)
No. of implants[a)]	66 (13.2±6.42)	91 (18.2±2.59)	70 (14.0±4.53)	61 (15.3±1.50)	76 (15.2±2.59)
No. of pre-implant loss[b)]	10 (13.2)	3 (3.19)	9 (11.39)	0	5 (6.17)
No. of total dead fetuses[c)]	2 (3.03)	8 (8.79)	3 (4.29)	2 (3.28)	2 (2.63)
Early resorptions	2 (3.03)	8 (8.79)	3 (4.29)	2 (3.28)	2 (2.63)
Late resorptions	0	0	0	0	1 (1.32)
Dead fetuses	0	0	0	0	0
No. of live fetuses[a)]	64 (12.8±6.76)	83 (16.6±2.88)	67 (13.4±4.77)	59 (14.8±0.96)	73 (14.6±2.79)
Sex ratio of live fetuses[d)]	1.37 (37/27)	0.8 (37/46)	0.68 (27/40)*	1.36 (34/25)	0.83 (33/40)
Body weight of live fetus (g)[e)]					
Male	5.38±0.87	4.99±0.63	5.1±0.41	5.06±0.22	4.86±0.46
Female	4.68±0.17	4.72±0.43	4..78±0.35	4.79±0.24	4.71±0.37
No. of live newborns with external anomalies	0	0	0	0	0
No. of live newborns with placentae anomalies	0	0	0	0	0

* $p<0.05$ (significantly different from control by χ^2 test).
[a)] Values in parentheses represent mean±S.D.
[b)] Values in parentheses represent percentages to the number of corpora lutea.
[c)] Values in parentheses represent percentages to the number of implants.
[d)] Values in parentheses represent number of male/female live fetuses.
[e)] Values are mean±S.D.

Table 2 Reproductive outcomes of dams treated orally with 2,3,7,8-TCDD on day 15 of gestation (terminal delivery)

Group and dose	Control	12.5 ng/kg	50 ng/kg	200 ng/kg	800 ng/kg
No. of dams	12	12	9	11	12
Gestational days[a)]	22.1±0.29	22.3±0.49	22.4±0.53	22.1±0.30	22.1±0.51
No. of implantations[b)]	200 (16.7±2.31)	178 (14.8±1.99)	131 (14.6±3.50)	179 (16.3±2.87)	199 (16.6±1.62)
No. of litter[b)]	188 (15.7±2.10)	159 (13.3±2.80)	125 (13.9±3.62)	160 (14.6±3.53)	175 9 (14.9±1.38)
Gestation index[c)]	100	100	100	100	100
No. of live newborns[b)]	186 (15.5±2.15)	159 (13.3±2.80)	125 (13.9±3.62)	160 (14.6±3.53)	175 (14.6±1.73)
Birth index[d)]	93.00	89.33	95.42	89.39	87.94
Sex ratio of live newborns[e)]	1.19 (101/85)	0.77 (69/90)*	0.67 (50/75)*	1.19 (87/73)	1.19 (95/80)
Body weight of live newborns (g)[a)]					
Male	5.8±0.4	6.1±0.4	6.4±0.8	6.2±0.7	5.8±0.3
Female	5.6±0.4	5.8±0.5	6±0.7	5.9±0.6	5.3±0.3
No. of stillborns[f)]					
Male	1	0	0	0	2
Female	1	0	0	0	2
Total	2 (1.06)	0	0	0	4 (2.23)
No. of live newborns with external anomalies	0	0	0	0	0

* $p<0.05$ (significantly different from control by χ^2 test).
[a)] Values are mean±S.D.
[b)] Values in parentheses represent mean±S.D.
[c)] Gestation index=(number of females with live newborns/Number of pregnant females)×100.
[d)] Values represent percentages to the number of implants.
[e)] Values in parentheses represent number of male/female live newborns.
[f)] Values in parentheses represent percentages to the number of litters.

number of implantations, the number of litter and the number of live newborns were lower compared to control in the 12.5 and 50 ng/kg group but not statistically significant. No significant differences were observed between TCDD-exposed animals and the vehicle-control group for other indices, i.e., gestational days, birth index (rate of live birth to number of implantations), number of still births, or body weight of live fetuses.

Pubertal data

Body weight changes of male and female offspring are shown in Fig. 1A and 1B, respectively. During lactation, body weight gain tended to be suppressed in male and female offspring in the 800 ng/kg group. After weaning, body weight gain was significantly suppressed in males of 800 ng/kg group on PND28–63, and in females of 800 ng/kg group on PND28–56.

Developmental parameters

Cumulative frequencies for eye opening are shown in Fig. 2. A significant delay of eye opening was observed in females of the 50 ng/kg group.

Cumulative frequencies for preputial separation in males

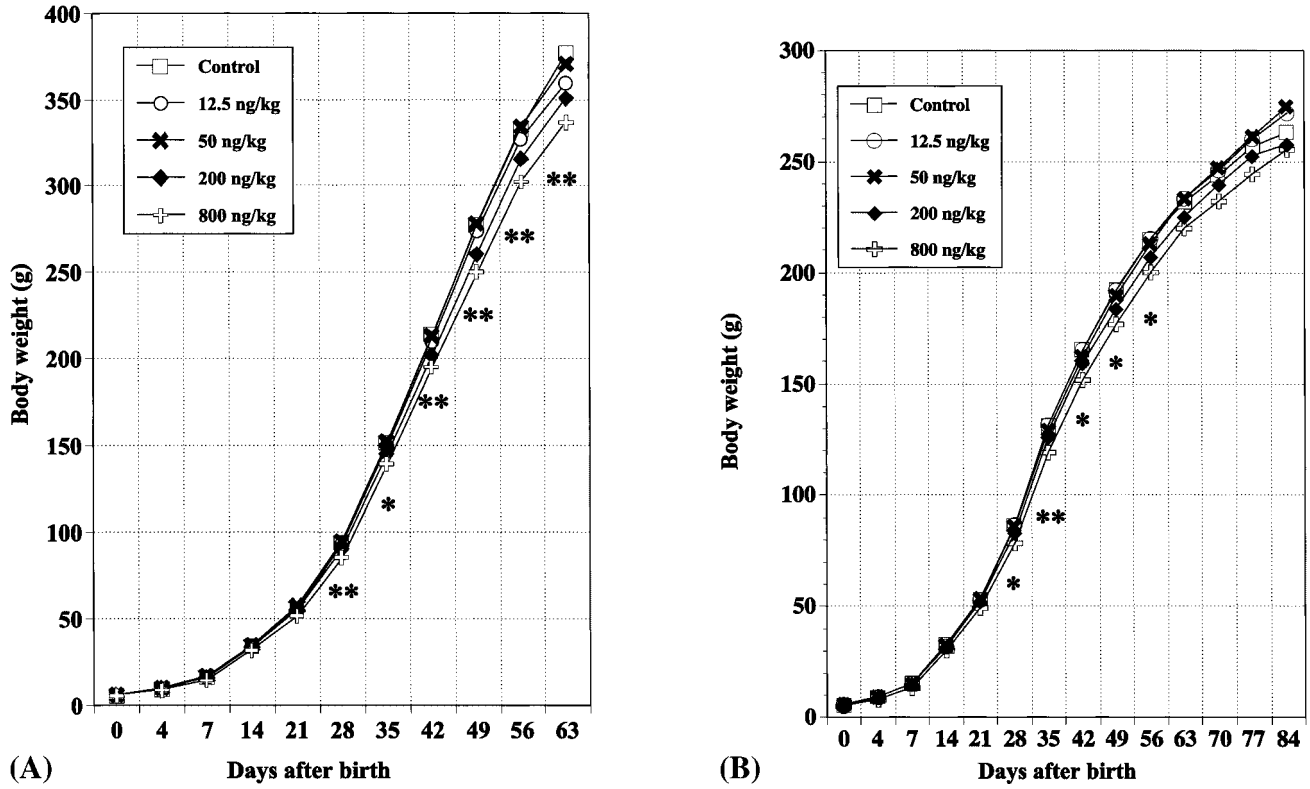

Fig. 1 Body weight change in male (A) and female (B) offspring from dams treated orally with TCDD on GD15. (A) Body weight gain was significantly suppressed in 800 ng/kg group on PND28, 42, 49, 56, 63 ($p<0.01$) and on PND35 ($p<0.05$) by Dunnett's multi-comparison test. (B) Significant suppression in body weight gain was observed in 800 ng/kg group on PND28, 42, 49, 56–63 ($p<0.05$) and on PND35 ($p<0.01$), in comparison to vehicle-treated control group by Dunnett's multi-comparison test.

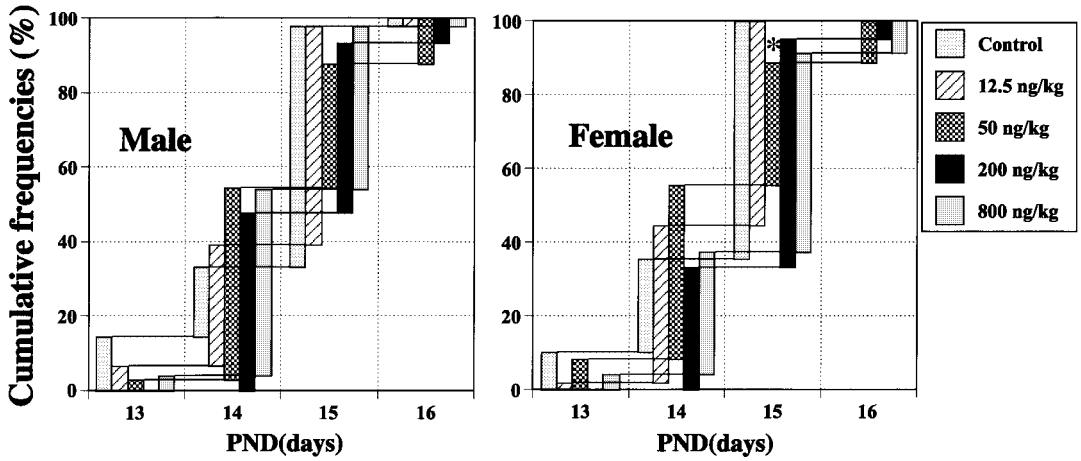

Fig. 2 Effect of *in utero* and lactational exposure to TCDD on eye opening. Significant delay of eye opening was observed in females of 50 ng/kg group (* $p<0.05$) by Wilcoxon's rank sum test.

are shown in Fig. 3. A significant delay in preputial separation was observed in the 200 and 800 ng/kg groups.

Figure 4 shows cumulative frequencies for vaginal opening. A significant delay in vaginal opening was observed in the 800 ng/kg group.

No evidence for treatment-related effects was found in the cyclicity (the length and frequency) of the estrus cycle.

Anogenital distance and spermatogenesis

The average anogenital distance of offspring on PND49 and PND63 varied from 24.4 mm to 27.3 mm and 26.5 mm to 28.8 mm, respectively, indifferent from TCDD treatment, no treatment-related effects was found (data not shown).

The number of average cauda epididymal sperm count ranged from 0.93 to 5.00×10^6/g tissue and 454 to 498×10^6/g tissue for PND49 and PND63, respectively, and no treatment-related effect was observed in the ejaculated sperm count examined between 12 and 13 weeks after birth. (data not shown).

Organ weight

On GD21, the absolute and relative liver weight was higher in the female fetuses in the 200 and 800 ng/kg groups

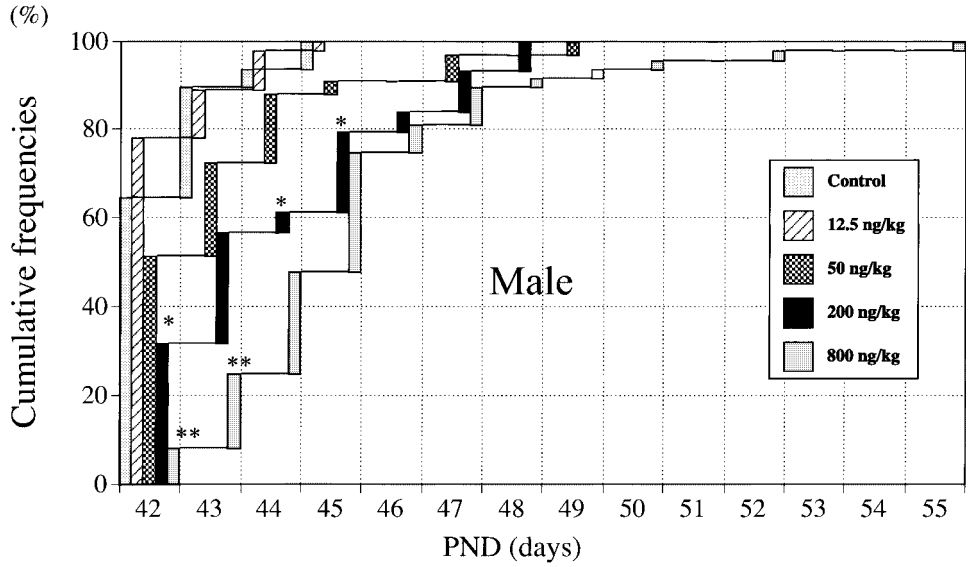

Fig. 3 Effect of *in utero* and lactational exposure to TCDD on male pubertal development (days at preputial separation). Significant difference from vehicle-treated control in cumulative frequencies of preputial separation was observed in males of 200 ng/kg group on PND42, 44 and 45, and in males of 800 ng/kg on PND42 and 43 (* $p<0.05$, ** $p<0.01$) by Wilcoxon's rank sum test.

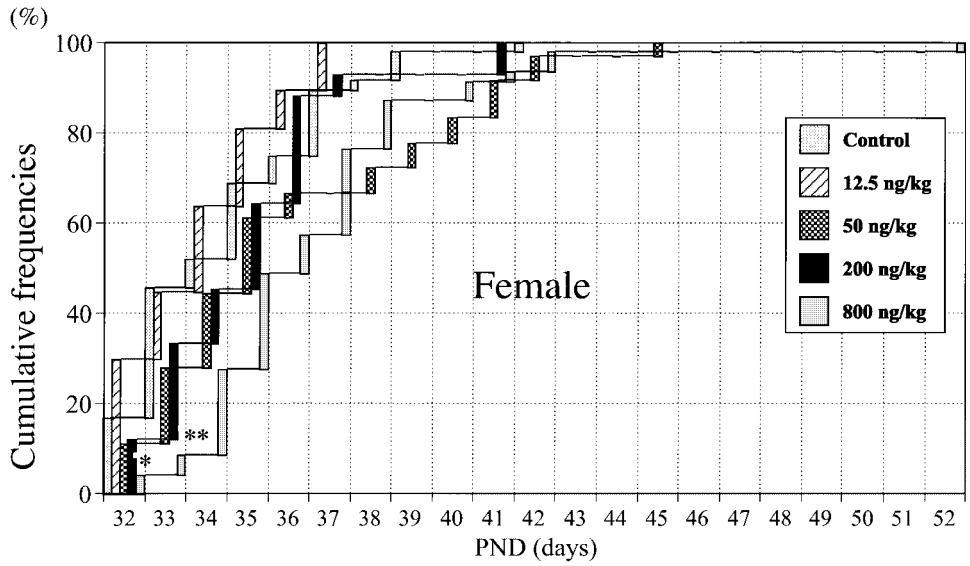

Fig. 4 Effect of *in utero* and lactational exposure to TCDD on female pubertal development (days at vaginal opening). Significant difference from vehicle-treated control in cumulative frequencies of vaginal opening was observed in females of 800 ng/kg group on PND32 and 33 (* $p<0.05$, ** $p<0.01$) by Wilcoxon's rank sum test.

(data not shown). On PND49, a significant decrease in the absolute weight of the ventral prostate was observed (Table 3). The relative liver weight was increased in the 12.5 and 800 ng/kg groups (data not shown). On PND63, no treatment-related effects were observed in either absolute or relative weight of male reproductive organs (Table 3). No treatment-related histopathological changes were observed at any time of examination in male and female offspring (data not shown).

Distribution study

Gestation

The tissue concentrations of radioactivity in dams and fetuses on GD16 and GD21 are summarized in Table 4 and Table 5, respectively. On GD16, the highest TCDD concentration was found in the liver, followed by the adrenal glands, lung, thymus, and adipose tissue (Table 4). There was a dose-dependent increase in the amount (% dose/tissue) of radioactivity in the liver. The liver contained approximately 12–29% of the administered dose of 12.5 to 800 ng TCDD/kg, which resulted in a tissue concentration of 37–5534 pg TCDD/g tissue. In contrast to the amount (% dose/tissue) of TCDD in the liver, TCDD amount in adipose tissue was decreased dose-dependently, which resulted in a dose-dependent increase in the liver to fat ratios.

Radioactivity was also found in embryos. The concentrations of TCDD in embryos were 12 and 52 pg TCDD/g embryo following exposure to 200 and 800 ng TCDD/kg, respectively. The ratios of embryo to maternal plasma ranged from 0.3 (200 or 800 ng TCDD/kg) to 1.0 (12.5 ng TCDD/kg). The maternal

Table 3 Absolute and relative weight of male reproductive organs

		Absolute weight (g) [Relative weight g/100 g B.W.]				
	Dose	0	12.5 ng/kg	50 ng/kg	200 ng/kg	800 ng/kg
PND49	Testes	2.63±0.31 [1.11±0.12]	2.49±0.17 [1.08±0.09]	2.56±0.2 [1.1±0.05]	2.55±0.17 [1.1±0.11]	2.45±0.18 [1.11±0.06]
	Epididymides	0.28±0.03 [0.12±0.02]	0.29±0.04 [0.13±0.02]	0.27±0.05 [0.11±0.02]	0.28±0.04 [0.12±0.01]	0.28±0.03 [0.13±0.01]
	Ventral Prostate	0.33±0.06 [0.14±0.03]	0.31±0.07 [0.13±0.03]	0.29±0.08 [0.13±0.03]	0.27±0.08 [0.12±0.03]	0.25±0.04* [0.11±0.02]
	Seminal Vesicle	0.57±0.09 [0.24±0.04]	0.6±0.09 [0.26±0.03]	0.56±0.16 [0.24±0.06]	0.53±0.12 [0.23±0.04]	0.48±0.12 [0.22±0.05]
PND63	Testes	3.18±0.24 [0.98±0.11]	3.22±0.31 [0.97±0.12]	3.34±0.18 [0.98±0.06]	3.32±0.17 [1.02±0.1]	3.22±0.4 [1.03±0.09]
	Epididymides	0.67±0.05 [0.21±0.02]	0.65±0.08 [0.2±0.03]	0.65±0.06 [0.19±0.03]	0.63±0.06 [0.19±0.02]	0.63±0.09 [0.2±0.02]
	Ventral Prostate	0.51±0.09 [0.16±0.03]	0.48±0.08 [0.15±0.02]	0.5±0.05 [0.15±0.01]	0.5±0.07 [0.15±0.02]	0.51±0.09 [0.16±0.03]
	Seminal Vesicle	1.07±0.17 [0.33±0.06]	1.14±0.19 [0.34±0.06]	1.15±0.13 [0.34±0.04]	1.08±0.19 [0.33±0.05]	1.04±0.17 [0.33±0.05]

Values are wean±S.D.
* $p<0.05$ (significantly different from control by Dunnett's multi-comparison test)

Table 4 Tissue concentration of radioactivity in dams on Day 16 of gestation

	Concentration (ng equiv. of 2,3,7,8-TCDD/mL or g) [% of dose/tissue]			
	12.5 ng/kg	50 ng/kg	200 ng/kg	800 ng/kg
Plasma	0.002±0.001 (1.0) [—]	0.010±0.003 (1.0) [—]	0.040±0.005 (1.0) [—]	0.171±0.056 (1.0) [—]
Brain	N.D.	0.005±0.002 (0.5) [0.05±0.02]	0.011±0.003 (0.3) [0.03±0.01]	0.051±0.019 (0.3) [0.03±0.01]
Thymus	0.012±0.002 (6.0) [0.08±0.02]	0.037±0.009 (3.7) [0.05±0.01]	0.093±0.016 (2.3) [0.03±0.01]	0.395±0.070 (2.3) [0.04±0.01]
Lung	0.014±0.001 (7.0) [0.35±0.04]	0.046±0.012 (4.6) [0.26±0.05]	0.121±0.047 (3.0) [0.18±0.06]	0.559±0.078 (3.3) [0.22±0.03]
Liver	0.037±0.010 (18.5) [11.82±3.52]	0.180±0.035 (18.0) [14.21±2.42]	0.793±0.094 (19.8) [16.98±2.21]	5.534±1.065 (32.4) [28.87±4.91]
Adrenal	0.018±0.004 (9.0) [0.03±0.00]	0.063±0.021 (6.3) [0.02±0.01]	0.169±0.058 (4.2) [0.02±0.01]	0.755±0.211 (4.4) [0.02±0.01]
Adipose	0.004±0.000 (2.0) [3.65±1.12]	0.014±0.004 (1.4) [2.78±0.77]	0.032±0.007 (0.8) [1.79±0.40]	0.139±0.033 (0.8) [2.49±0.38]
Placenta	0.002±0.001 (1.0) [—]	0.009±0.002 (0.9) [—]	0.020±0.007 (0.5) [—]	0.079±0.016 (0.5) [—]
Embryo	0.002±0.000 (1.0) [0.12±0.05]	0.005±0.002 (0.5) [0.13±0.08]	0.012±0.002 (0.3) [0.07±0.02]	0.052±0.014 (0.3) [0.09±0.05]
Liver to Adipose ratios	3.1	4.9	8.6	10.8

Each value represents the mean±S.D. of five rats.
( ): tissue/plasma ratio, —: not calculated, N.D.: not detected.

body burdens estimated from the percent dose/tissue (except skin) were 2.23, 11.1, 45.3 and 290 pg TCDD/g in the 12.5, 50, 200, and 800 ng/kg group, respectively.

On GD21, the highest TCDD concentration was found in the adipose tissue followed by liver, adrenal, thymus, and lung in the 12.5 and 50 ng/kg groups, while the liver still contained the highest concentration following exposure to 200 and 800 ng TCDD/kg (Table 5). The liver concentration was decreased from that on GD16, while the adipose tissue concentration was increased from that on GD16. A dose-dependent increase and decrease in the amount of tissue content (% of dose/tissue) in liver and adipose tissue, respectively, were also observed on GD21. The liver to fat ratios were also increased dose-dependently. However, the ratios were smaller on GD21 than on GD16. The estimated maternal body burdens on GD21 in the 12.5, 50, 200, and 800 ng/kg group were 1.31, 7.05, 28.8 and 150 pg TCDD/g, respectively. Among fetal tissues, relatively high concentrations of TCDD were found in the lung and liver. The ratio of fetal lung to maternal plasma ranged from 1.3 (800 ng TCDD/kg) to 3.3 (50 ng TCDD/kg). The ratio of tissue

Table 5 Tissue concentration of radioactivity in dams on Day 21 of gestation

	Concentration (ng equiv. of 2,3,7,8-TCDD/mL or g) [% of dose/tissue]			
	12.5 ng/kg	50 ng/kg	200 ng/kg	800 ng/kg
Plasma	N.D.	0.003±0.001 (1.0)	0.016±0.007 (1.0)	0.170±0.031 (1.0)
		[—]	[—]	[—]
Brain	N.D.	0.002±0.001 (0.7)	0.003±0.008 (0.2)	0.016±0.019 (0.3)
		[0.01±0.01]	[0.01±0.00]	[0.01±0.01]
Thymus	0.005±0.002	0.020±0.002 (6.7)	0.066±0.019 (4.1)	0.214±0.074 (3.1)
	[0.02±0.01]	[0.02±0.00]	[0.02±0.01]	[0.01±0.01]
Lung	0.003±0.001	0.017±0.005 (5.7)	0.057±0.008 (3.6)	0.224±0.073 (3.2)
	[0.08±0.01]	[0.10±0.03]	[0.09±0.01]	[0.08±0.02]
Liver	0.008±0.003	0.062±0.010 (20.7)	0.353±0.094 (19.8)	2.095±0.439 (29.9)
	[2.75±1.02]	[4.47±0.44]	[6.19±1.37]	[10.41±3.59]
Adrenal	0.007±0.002	0.028±0.005 (9.3)	0.072±0.019 (4.5)	0.314±0.102 (4.5)
	[0.01±0.00]	[0.01±0.00]	[0.01±0.00]	[0.01±0.00]
Adipose	0.020±0.006	0.083±0.012 (27.7)	0.233±0.051 (14.6)	1.026±0.238 (14.7)
	[7.46±2.32]	[7.50±0.98]	[5.45±1.29]	[5.95±1.32]
Placenta	N.D.	0.004±0.002 (1.3)	0.011±0.005 (0.7)	0.052±0.015 (0.7)
		[—]	[—]	[—]
Fetal liver	N.D.	0.003±0.000 (1.0)	0.013±0.002 (0.8)	0.082±0.093 (1.2)
		[0.06±0.02]	[0.08±0.02]	[0.15±0.18]
Fetal lung	0.002±0.001	0.010±0.003 (3.3)	0.034±0.007 (2.1)	0.092±0.022 (1.3)
	[0.07±0.02]	[0.08±0.04]	[0.08±0.02]	[0.06±0.02]
Liver to Fat ratio	0.4	0.75	1.5	2.0

Each value represents the mean±S.D. of five rats.
( ): tissue/plasma ratio, —: not calculated, N.D.: not detected.

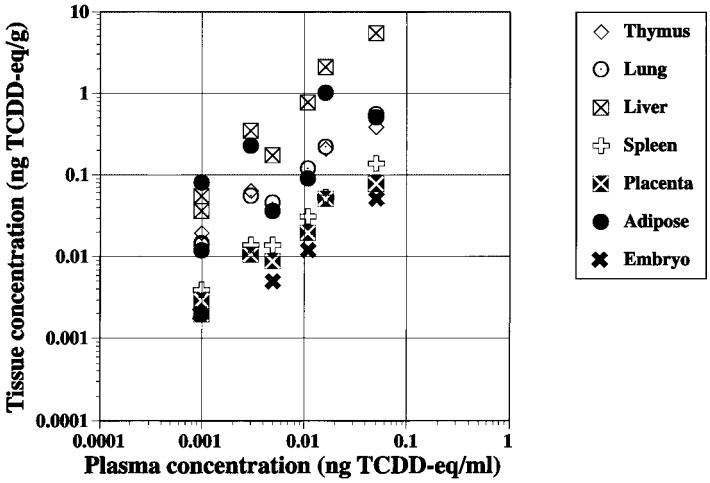

Fig. 5 The ratios of maternal tissues to maternal plasma TCDD concentration in pregnant rats (GD16 and GD21).

to maternal plasma in pregnant rats was the highest in the liver, followed by adipose tissue, adrenal glands, lung, and thymus (Fig. 5). The ratios of placenta and embryo to maternal plasma were less than one (Fig. 5).

C_{max} (peak concentration) for adipose tissue was observed on GD21, while C_{max} for other tissues were observed on GD16.

Post-weaning

The highest concentration was found in adipose tissue, followed by the liver and thymus of offspring on PND49, 63, and 70 (Fig. 6). The concentration in other tissues was under the detection limit, except for a very low concentration in the lung on PND49.

Non-pregnant females

The tissue distribution pattern of TCDD in non-pregnant female rats on one day after or 6 days after dosing was similar to that of pregnant rats on GD16 or GD21, respectively. One day after dosing, the highest concentration was found in the liver, followed by the adrenal glands, adipose tissue, lung, and thymus (Table 6). A tendency toward the dose-dependent increase and decrease in liver content (% dose/tissue) and adipose content, respectively, was also observed in non-pregnant female rats. The liver concentration in non-pregnant female rats was higher than that in pregnant rats. At 6 days after dosing, the highest concentration was still found in the liver in the 200 and 800 ng TCDD/kg groups (data not shown).

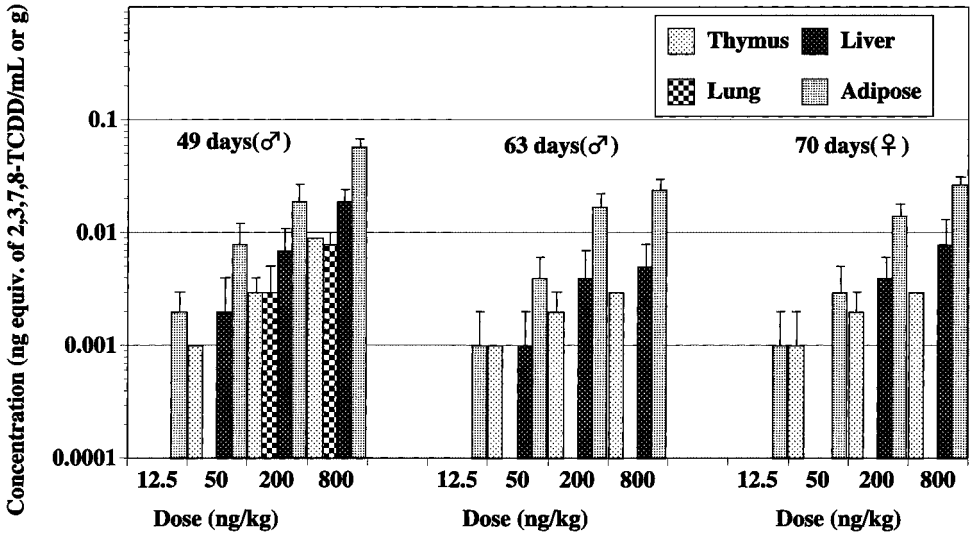

Fig. 6 Tissue concentration of radioactivity in offspring on PNDs 49, 63 and 70.

Table 6 Tissue concentration of radioactivity in non-pregnant rats 1 day after oral treatment with ^{3}H-2,3,7,8-TCDD

	Concentration (ng equiv. of 2,3,7,8-TCDD/mL or g) [% of dose/tissue]			
	12.5 ng/kg	50 ng/kg	200 ng/kg	800 ng/kg
Plasma	0.001±0.001 (1.0)	0.008±0.003 (1.0)	0.044±0.013 (1.0)	0.134±0.091 (1.0)
	[0.54±0.50]	[0.79±0.35]	[1.10±0.35]	[0.74±0.52]
Brain	N.D.	0.004±0.002 (0.5)	0.009±0.004 (0.2)	0.035±0.014 (0.3)
	N.D.	[0.06±0.02]	[0.03±0.01]	[0.03±0.01]
Thymus	0.010±0.002 (10.0)	0.035±0.006 (4.4)	0.113±0.013 (2.6)	0.263±0.043 (2.0)
	[0.09±0.03]	[0.07±0.02]	[0.00±0.00]	[0.03±0.01]
Lung	0.014±0.003 (14.0)	0.050±0.011 (6.3)	0.183±0.078 (4.2)	0.329±0.151 (2.5)
	[0.41±0.09]	[0.36±0.10]	[0.32±0.13]	[0.16±0.07]
Liver	0.046±0.016 (46.0)	0.281±0.076 (35.1)	1.453±0.348 (33.0)	6.124±1.807 (45.7)
	[11.92±3.97]	[18.71±5.35]	[23.87±4.89]	[29.71±7.09]
Adrenal	0.020±0.005 (20.0)	0.059±0.017 (7.4)	0.251±0.083 (5.7)	0.404±0.201 (3.0)
	[0.03±0.01]	[0.03±0.01]	[0.03±0.01]	[0.02±0.01]
Adipose	0.014±0.004 (14.0)	0.054±0.024 (6.8)	0.211±0.082 (4.8)	0.616±0.118 (4.6)
	[4.10±1.19]	[3.96±1.73]	[3.85±1.50]	[2.91±0.60]
Ovary	0.006±0.002 (6.0)	0.020±0.006 (2.5)	0.068±0.021 (1.5)	0.118±0.060 (0.9)
	[0.02±0.01]	[0.02±0.01]	[0.01±0.00]	[0.01±0.01]
Liver to Fat ratio	3.3	5.2	6.9	9.9

Each value represents the mean±S.D. of five rats.
( ): tissue/plasma ratio, —: not calculated, N.D.: not detected.

A dose-dependent increase in the liver to adipose tissue ratios (0.58–3.19) was observed, and these ratios were higher than those observed on GD21 (0.4–2.0). At 27 days after dosing, the highest concentration was found in adipose tissue, except in the 800 ng TCDD/kg group, in which the liver had the highest concentration (data not shown).

Discussion

Dose-response study

Several research groups investigated how *in utero* and lactational exposure to TCDD affected growth and development of reproductive organs in rat offspring using very similar experimental protocols (7–11). Pregnant rats were given a single oral dose of TCDD on GD15 and allowed to deliver and foster until weaning. The endpoints that were affected by TCDD and the LOAELs of TCDD for those endpoints were different among studies. Using Holtzman strain, Peterson and his associates observed the following effects at the LOAEL as indicated in the parenthesis: reduced testis weight (64 ng TCDD/kg), reduced ventral prostate weight (64 ng TCDD/kg), reduced daily sperm production (DSP) (64 ng TCDD/kg), reduced cauda epididymal sperm count (64 ng TCDD/kg), reduced anogenital distance (AGD) (160 ng TCDD/kg), reduced plasma testosterone level (1000 ng TCDD/kg), and demasculinization of male sexual behavior (64 ng TCDD/kg) (7–9). Gray's group (10) conducted a study using LE rats and observed reduced DSP, reduced cauda epididymal sperm count, and reduced AGD, but at higher doses than those reported by Mably et al. (7). They did not find reduced testis weight or reduced plasma testosterone level (10).

We also conducted a study using Holtzman rats and observed significantly reduced AGD at a dose as low as 50 ng TCDD/kg and significantly reduced ventral prostate weight at 200 ng TCDD/kg, but did not observe changes in sperm count at any site, DSP, or testis weight (11). In the present study, the effects of TCDD on the male reproductive endpoints were minimum compared with those in previous studies using the same experimental protocol (7, 8, 10, 11). As far as the male reproductive endpoints are concerned, we observed a decrease in the absolute but not the relative weight of the ventral prostate at the highest dose (800 ng TCDD/kg). Although the effective dose was different, reduced ventral prostate weight was sensitive and consistent male reproductive endpoint among studies after *in utero* and lactational exposure to TCDD (7, 10, 11).

The apparent inconsistency of the effects on male reproductive endpoints in response to TCDD might be partly due to a strain difference. Strain difference between Han/Wistar and LE rats in sensitivity to dioxin-induced lethality and certain male reproductive effects such as sperm numbers are ascribed to genetic differences in C-terminal transactivation domain of AhR (14). No such differences were reported between Holtzman and LE strain of rats. There is more than a 30-fold difference in the inducibility of hepatic CYP1A1 mRNA in response to TCDD exposure among rat strains (15). The relative hepatic CYP1A1 mRNA expression at 72 hr after a single oral dose of TCDD (40 ng/kg) in Holtzman rats was about two times higher than that of LE rats (10). However, there is still inconsistency between studies using the same rat strain, such as Holtzman rats in the studies of Mably and Ohsako (7, 8, 11) and LE rats in the studies of Gray (10) and the present study. Other factors other than strain difference might be involved in these inconsistencies. As neither Holtzman rats nor LE rats are inbred strains, there is no guarantee that the genetic background is maintained over time. Another possible explanation for the inconsistency is that the precise embryonic age of TCDD exposure might be slightly different among studies. If the critical window of sensitivity for each endpoint was narrow and different, even a small difference in embryonic age at the time of TCDD administration might have resulted in a different outcome. Peterson's and Gray's groups used pregnant rats from commercial breeders, while, in the Ohsako study and the present study, pregnant rats were obtained by mating a female in the proestrus with a male rat for one night. If commercial breeders produced pregnant rats by having female rats cohabit with a male rat until they found a vaginal plug, the actual time of fertilization might differ from that of one-night mating. As fetuses/offspring are exposed to TCDD indirectly via placentas and through lactation, many factors such as distribution volume of dams, rate of placental transfer, TCDD concentration in milk, etc., could be involved in the manifestation of the various endpoints.

In the female offspring of LE rats exposed to TCDD perinatally, malformations in external genitalia were observed, such as cleft phallus and vaginal thread (16). We could not find such malformations in female offspring in the present study. The inconsistency among studies must be further clarified since WHO TDI depends on the male and female reproductive endpoints as described above.

Pubertal development

In utero and lactational TCDD exposure resulted in a significant delay in preputial separation at 200 and 800 ng TCDD/kg and in vaginal opening at 800 ng TCDD/kg in the present study. At the dose of 800 ng/kg, delayed pubertal development was associated with lower body weight gain, suggesting the possibility that the delayed puberty was merely a reflection of delayed physical development, since the body weight at puberty (body weight on the day of preputial separation or vaginal opening) was not different among exposed groups. A significant delay in preputial separation was reported at 200 and 800 ng TCDD/kg, and a delay in vaginal opening was noted at 800 ng TCDD/kg in LE rats (10, 16). Vaginal opening was also significantly delayed at 400 ng TCDD/kg in Holtzman rats (17). In contrast to these findings, accelerated vaginal opening and onset of estrus were noted after *in utero* and lactational exposure to 800 ng TCDD/kg in LE rats (18). These discrepancies need further investigation.

Sex ratio

Since accidental TCDD exposure has been reported to show a drastic decrease in the sex ratio (male/female) in Seveso residents in Italy (19), we examined the sex ratio of fetuses or pups in the present study. The sex ratio was significantly reduced at 50 ng TCDD/kg on GD21 and at 12.5 and 50 ng TCDD/kg at birth, but not at other doses. The possibility of the low-dose effects of TCDD could not be excluded, since two sets of experiments (necropsy on GD21 and at birth) showed the same tendency of reduced sex ratio at low doses. Although not statistically significant, the number of implantations, the number of litter and the number of live newborns were also lower in low doses (12.5 and 50 ng/kg). An inverted U-shape dose response was observed in the ventral prostate weight of mice offspring exposed *in utero* to a synthetic estrogen, diethylstilbestrol (DES) (20). As TCDD is also an endocrine disruptor, it is possible that the endocrine effects of TCDD could occur only within a certain range of dosage. In fact, low-dose effects were reported in the behavior of rats exposed perinatally to a low dose of TCDD (21, 22).

Distribution study

Pregnant rats

Hurst et al. conducted a distribution study in pregnant LE rats using the same protocol as that in the present study (12). Amounts (% dose/tissue) in organs on GD16 at the dose of 50, 200, and 800 ng TCDD/kg were 34.1, 30.1, and 47.1 for the liver and 10.6, 6.29, and 6.92 for the adipose tissue, respectively. In the present study, they were 14.2, 17.0, and 28.9 for the liver and 2.78, 1.79, and 2.49 for the adipose tissue, respectively. The amounts found in the liver and adipose tissue were lower compared to those reported by Hurst et al. (12) although we do not have an explanation for the difference between the two studies. The liver to fat ratios at doses of 50, 200, or 800 ng TCDD/kg were 4.9, 8.6, or 10.8, respectively, which were comparable to those reported by Hurst et al. (5.7, 8.2, or 10.9, respectively). These consistent data may suggest that the distribution pattern in the present study was similar to that in the

study by Hurst et al. (12). The fetal concentrations at doses of 50, 200, or 800 ng TCDD/kg were 5, 12, or 52 pg TCDD/g tissue, respectively, which were comparable to those of 5.3, 13.2, or 39.1 pg TCDD/g tissue observed in Hurst's study. On GD21, the amounts (% dose/tissue) in adipose tissue were increased, but they were decreased in other tissues. These changes could be partly explained by a faster elimination half-life in the liver than in adipose tissue (23). The highest concentration was still observed in the liver at a dose of 200 and 800 ng TCDD/kg. The distribution pattern on GD21 was close to that observed in Hurst's study (12).

A tendency toward a dose-dependent increase in the liver to fat ratios was observed, indicating that a larger amount of TCDD was accumulated in the liver following administration of higher doses (more than 200 ng/kg in the present study). CYP1A2 induction was reported to be involved in the mechanism of TCDD sequestration in the liver (24, 25). Amounts (% dose/tissue) in fetal liver were also increased dose-dependently, indicating the same sequestration mechanism might also operate in fetal liver.

Though the ratios of tissue to maternal plasma concentration were different among tissues, the ratios were generally constant in the same tissue over the dose range, suggesting the different affinity of each tissue for TCDD. The highest tissue to maternal plasma ratio was found in the liver, followed by adipose tissue, thymus, and lung. The ratios for placentas and embryos were less than one, indicating that some kind of placental barrier may exist.

Non-pregnant rats

One day after dosing, the amounts (% dose/tissue) in the liver and adipose tissue tended to be higher than those observed in pregnant rats. On 6 day post-administration, the liver concentrations in non-pregnant female rats were higher at a dose of 200 and 800 ng TCDD/kg than those of pregnant rats. There was a tendency of a higher liver to fat ratio in non-pregnant rats than in pregnant rats. The feto-placental complex is thought to contribute to the distribution volume, which might reduce the sequestration of TCDD to the liver in pregnant rats.

As the effects of TCDD on the reproductive endpoints in the present study were minimum compared with previous studies using the same experimental protocol, we could correlate the body burdens of dams or fetuses/pups to only a few endpoints. Among the reproductive endpoints affected, the reduced ventral prostate weight was sensitive and consistent with previous studies using the same experimental protocol. Effects of *in utero* TCDD exposure on the development of ventral prostate were intensively studied (26–30). It is suggested that TCDD exposure impairs prostate growth and androgen responsiveness by inhibiting prostatic epithelial cell differentiation (28). Although the effective dose varied study to study, the reduced ventral prostate weight could be a candidate for the endpoint on which TDI was based since this effect was consistent among studies and was a very sensitive endpoint of developmental toxicity in response to TCDD exposure. For a better estimate of TDI, the affected endpoints should be presented with the body burdens of dams or fetuses/pups. In the present study, administration of TCDD on GD15 at a dose of 12.5, 50, 200, and 800 ng TCDD/kg resulted in a concentration of 2, 5, 12 and 52 pg/g in a single fetus, respectively on GD16. A dose of 800 ng TCDD/kg resulted in maternal body burden and fetal concentrations of 290 pg TCDD/g and 52 pg TCDD/g on GD16, respectively. This body burden corresponds to a reduced ventral prostate weight. Reduced prostate weight is a sensitive and commonly observed endpoint so that the body burdens of dams and fetuses at the LOAEL of this endpoint could be served as the basis for establishing TDI for dioxins.

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