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Antiestrogenic Effects of Low Doses of 2,3,7,8-TCDD in Offspring of Female Rats Exposed Throughout Pregnancy and Lactation

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In recent years considerable interest has developed in elucidating effects of endocrine-disrupting substances on male fertility after in utero and/or lactational exposure. Exposure to hormonally active chemicals during early development may alter permanently male reproductive function (Colborn et al. 1993). The male reproductive system of the rat is the most sensitive target of possible adverse effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The reproductive effects of TCDD on male offspring have been investigated (Mably et al. 1992 a, b, c; Gray et al. 1995; Theobald and Peterson, 1997; Faqi et al. 1998a), but only a few studies exist on effects of TCDD on female reproduction after in utero exposure. Exposure to a single dose of 1 µg TCDD on day 15 of gestation results in malformations in external genitalia of female rats (Gray and Ostby, 1995; Flaws et al. 1997). Repeated exposure using low dose levels of TCDD resembling the human situation is fundamental for risk assessment.

The purpose of the present study was to elucidate the reproductive effects of TCDD on female offspring of Wistar rats. In this study attempts were made to keep the TCDD body burden as constant as possible. For this reason the dams were treated with an initial loading dose followed by a weekly maintenance dose. The results of the reproductive effects of TCDD on male offspring were published separately (Faqi et al. 1998a).

MATERIALS AND METHODS

Female Wistar rats (Bor: spf, TNO; Fa. Winkelmann, Borchen, FRG) were adapted to the conditions of our animal quarters 2 weeks before starting the experiment. They were kept under specific conditions at a constant day/night cycle (light from 9:00 a.m. to 9:00 p.m.) at 21 ± 1°C and 50 ± 5% relative humidity. They received standard pellet feed (Altromin 1324 Lage, FRG) and tap water was offered as drinking water ad libitum.

^{14}C-TCDD supplied by Cambridge Isotope Laboratories (Woburn, MA, USA) had a radiochemical purity of 97% and a specific activity of 107 mCi/mmol (according to the manufacturer). TCDD was dissolved in a mixture of

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toluene/DMSO (1:2, v/v) in order to prepare a solution suitable for subcutaneous injection (Abraham et al. 1988) and was administered with a 100 µl Hamilton microsyringe under the skin of the back(0.1 ml/kg body wt). Female rats (n =100) were randomly assigned to 25 rats/group, one of which served as a vehicle control, the other 3 groups were treated with three different dose regimens of TCDD. The rats were treated subcutaneously 2 weeks prior to mating, and during mating, pregnancy, and throughout the lactation period. They received an initial loading dose of 25, 60 or 300 ng TCDD/kg body wt, followed by a weekly maintenance dose of 5, 12 or 60 ng TCCD/kg body wt (here referred to as TCDD 25/5, TCDD 60/12, and TCDD 300/60). The animals were treated between 8:00-9:00 a.m. The subcutaneous route was used to assure complete absorption (Abraham et al. 1988; Hagenmaier et al. 1990). The loading and maintenance doses were calculated using established pharmacokinetic estimations considering 3 weeks as the half-life of TCDD in rats. This form of exposure paradigm has been proven to be suitable by Krowke at al. (1989) and was adapted in a series of studies conducted previously at our institute (Chahoud et al. 1989; Thiel et al. 1994; Koch et al. 1995). The weekly maintenance dose was about 20% of the loading dose.

The treated F_0 females (25/group) were mated with untreated males (1:1) 3 h daily for 8 days. Vaginal smears were collected daily and examined for the presence of sperm. The day of sperm detection in vaginal smears was considered as day 0 of pregnancy. Maternal weight was monitored throughout gestation, while individual pups were weighed on postnatal days (PND) 2, 7, 14, and 22. Pups were weaned at PND 22 and housed in groups by sex and litter. The reproductive effects of TCDD on male offspring were investigated on PND 70 or 170 which corresponds to the pubertal and adulthood stages of development and the results were published separately (Faqi et al. 1998a).

Three dams (F_0 females) in each group were killed on gestation day 21 and their fetuses removed. The concentration of TCDD in the maternal liver and fat was measured. Samples of pooled liver from each litter were obtained and the liver concentration of the TCDD determined. Likewise, at weaning (PND 22) the concentration of TCCD in the liver of selected offspring (n = 6/group) was measured. Radioactivity was measured by scintillation counting (LKB 1217 Rack Beta, Freiburg, Germany) following solubilization of tissue samples in 3 ml TS-1 (Zinsser, Frankfurt/M., Germany), sonication for 30 min 2 days later and addition of about 15 ml scintillation cocktail (Hionic-Fluor, Packard, USA). The data were corrected for quenching (Abraham et al. 1988). TCCD is known to be metabolically stable and polar metabolites are excreted rapidly. The measured radioactivity essentially represents the unchanged ^{14}C-TCDD (Rose et al. 1976; Abraham et al. 1988).

All F_1 females were examined daily for vaginal opening starting at PND 35 until vaginal opening had been completed in all the females. On PND 60, daily vaginal smears were obtained from each animal to detect the effect of in utero and lactational TCDD exposure on the estrous cycle. The estrous cycle of each female was monitored for 21 consecutive days. The vaginal smears were classified as

either proestrous, estrous, metestrous, or diestrous. The number of vaginal smears investigated for sexual length was 294 in the control, 210 in the TCDD 25/5, 199 in the TCDD 60/12 and 225 in the TCDD 300/60 group. Those investigated for estrous length was 352 in the control, 243 in the TCDD 25/5, 240 in the TCDD 60/12 and 277 in the TCDD 300/60 group. For the diestrous length, the number of vaginal smears was 326 in the control, 251 in the TCDD 25/5, 200 in the TCDD 60/12 and 234 in the TCDD 300/60 group. On PND 220, the F_1 females were split into two cohorts; one cohort was used for fertility study and the other cohort was killed during the estrous stage. In those rats killed during the estrous stage the ovaries and uterus were removed and weighed. The ovary was immediately fixed in Bouin's solution for 1-2 days, then embedded in paraffin, sectioned at 5 µm, stained with hematoxylin/eosin, and examined for histological lesions. The histological lesions were examined qualitatively. For the fertility study, the F_1 females were mated with untreated male rats (1:1) 3 hours daily for 2 weeks. The day of sperm detection in vaginal smears was considered as day 0. The dams were sacrificed at day 21 post conception and implantation sites, viable and dead fetuses, resorptions as well as external malformations were enumerated and recorded. In addition the mating, pregnancy and fertility indices were calculated. In the following the definition of terms used is given:

Mating index: no. of females made sperm positive/ no. of males mated x 100.

Pregnancy index: no. of males that made females pregnant/ no, of males that made females sperm positive x 100.

Fertility index: no. of days elapsed until the male animal had fertilized its female partner.

For statistical analysis, the variables pregnancy, fertility mating indices and sex ratio were analyzed using the Chi-square test. All other data were analyzed using the single factor analysis of variance (ANOVA) followed by a multiple comparison procedure as exemplified by the Tukey-test with equal or unequal sample sizes at a significance level of $p < 0.05$ (Gad and Weil, 1982).

RESULTS AND DISCUSSION

The dams (F_0) treated with low doses of TCDD did not shown signs of maternal toxicity such as mortality and weight loss (data not shown). The mating and fertility indices were not affected, however, the pregnancy index was significantly reduced in dams exposed to the highest dose of TCDD (Table 1). In Table 1, the mating days required up to pregnancy is reported. This shows that only few females from each dose group required more than 6 days to become pregnant and this was not statistically significant (see Table 1).

On gestational day 21, a considerable amount of TCDD was detected in maternal liver and fat. In the highest dose group, the concentration (ng/g) of TCDD in maternal fat was similar to that determined in the liver (Faqi et al. 1998a). The concentration of TCDD in the fetal liver on day 21 of gestation was under the limit of detection (0.08 ng/g). At weaning, the concentration of TCDD in the offspring liver was 0.24 ng/g, 0.39 ng/g and 1.78 ng/g,, in the TCDD 25/5, TCDD 60/12, and

TCDD 300/60 groups, respectively (Fig. 1). These concentrations correspond to about 0.7% of the maternal doses.

The mean age at vaginal opening was delayed in TCDD-exposed females, the results, however, were significant only in the TCDD 25/5 and TCDD 300/60 groups (Table 2). Nevertheless, the effect was not dose-dependent. Presenting the data as percent of F_1 females exhibiting vaginal opening per age interval, we will observe that on PND 38, 70% of the females had complete vaginal opening in the control group compared to 35% in the TCDD 300/60 group. On PND 39, the vaginal opening was completed in the control females, whereas in the TCDD 25/5 and TCDD 300/60 groups a complete vaginal opening was achieved in only 76 and 70% of the females, respectively (data not shown). The sexual cycle length was not significantly different between the groups. The mean number of days spent in estrous phase was significantly decreased in the lowest and in the middle dose groups. However, in the highest dose group the reduction was not significant. The diestrous length was comparable in all dose groups as well as the control group (Table 3). Moreover, the reproductive performance of these F_1 females was not affected in any group (data not shown). The ovary weights of the female offspring were not significant between the groups (Table 4) and no histological abnormalities including changes in the follicular, luteal, and interstitial compartments were observed in the ovary. However, the uterine weights were significantly reduced in the TCDD 300/60 group when compared to the control weights (see Table 4).

[a]**Table 1.** Mating, fertility, and pregnancy indices of dams exposed to TCDD during pregnancy and lactation. For the definition of the terms see Materials and Methods, *Note:* Mating days up to pregnancy indicates the mating days required until the female rat was made sperm positive

Groups	Mating/pregnancy index (%)		Fertility index (days) (X ± SD)	Mating days up to pregnancy 1 - 5 days		6 - 14 days	
				n	%	n	%
Control	96	100	3.3 ± 3.3	21	88	3	12
TCDD 25/5	96	100	3.5 ± 3.4	20	83	4	17
TCDD 60/12	92	91	3.6 ± 3.4	18	78	5	22
TCDD 300/60	100	84*	3.6 ± 3.1	19	76	6	24

* Values differ significantly from control values.

[a] Data adapted from Faqi et al. 1998a., n = number of animals

Prenatal and postnatal exposure to toxicants can produce changes that may not be predicted from effects seen in adults, and such effects are usually irreversible (Schardein, 1993; Colborn et al. 1993). The exposure scenario (repeated low dose exposures) of the present study provides a more meaningful low dose response that can be useful for risk assessment. This study has shown that the offspring were predominantly exposed via lactation rather than through placental transfer. This is consistent with the toxicokinetic study carried out in pregnant and lactating

Table 2. Effects of in utero and lactational TCDD exposure on age at vaginal opening. Vaginal opening was examined from day 36 to 42 postnatally.

Parameter	Control (n = 101)	TCDD 25/5 (n = 92)	TCDD 60/12 (n = 73)	TCDD 300/60 (n = 79)
Mean age at vaginal opening (days)	37.6 ± 0.8	38.5 ± 1.4*	37.9 ± 1.1	38.1 ± 1.3*

* = ($p < 0.05$), *Note*: data are Mean ± SD, n = number of animals

Table 3. Effects of in utero and lactational TCDD exposure on sexual cycle, estrous and diestrous length.

Parameters	Control	TCDD 25/5	TCDD 60/12	TCDD 300/60
Cycle length (days)	5.2 ± 0.9	5.2 ± 1.1	5.2 ± 1.2	5.2 ± 1.0
Estrus length (days)	1.1 ± 0.4	1.1 ± 0.2*	1.1 ± 0.2*	1.1 ± 0.3
Diestrus length (days)	1.2 ± 0.8	1.2 ± 1.0	1.2 ± 1.0	1.1 ± 0.6

*= ($p < 0.05$), *Note*: data are Mean ± SD.

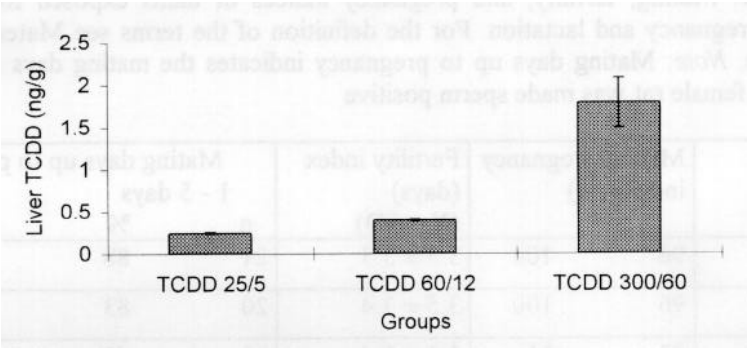

Figure 1. TCDD concentration (ng/g) in liver of offspring on PND 21.
Note: data are Mean ± SD

Sprague-Dawley rats which indicated that transfer through the placenta was very limited and only about 0.01% of the total dose was found in the fetuses liver (Li et al. 1995). Similarly, Koch et al. (1995) reported an effective elimination of TCDD via milk.

Vaginal opening was completed on PND 39 in the control, this was delayed to PND 42 in offspring females exposed to TCDD 25/5 and TCDD 300/60. Vaginal opening was delayed by several days in F_1 females after in utero and lactational 2,3,7,8-TCDD exposure (Thiel et al. 1994). Likewise, exposure to PCB 126 (a

Table 4. Effects of in utero and lactational TCCD exposure on body, ovary and uterine weights of F_1 females at adulthood.

Weights	Control (n = 20)	TCDD 25/5 (n = 20)	TCDD 60/12 (n = 19)	TCDD 300/60 (n = 17)
Body weight (g)	239.0 ± 23.2	245.2 ± 18.8	249.7 ± 22.8	241.8 ± 22.1
Ovary (g)	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
Uterus (g)	0.8 ± 0.3	0.7 ± 0.2	0.9 ± 0.3	0.6 ± 0.2*

*=$p < 0.05$, n = number of animals, *Note:* data are Mean ± SD

non-ortho coplaner dioxin-like congener) on day 15 of gestation induced a delay in vaginal opening in female pups by more than a week (Faqi et al. 1998b). Vaginal opening results from an increase in the blood level of estradiol and the age at which this occurs is the most commonly measured marker of puberty. Exposure to 10 mg raloxifene/kg/d was reported to accelerate the vaginal opening by 2 days. When these females were mated with treated males (1:1) a decrease in mating, fertility and litter size was reported (Buelke-Sam et al. 1998). Moreover, adverse reproductive outcomes (slight decrease in fertility) have been reported in rodents when preputial separation is delayed by 7-10 days (Van den Dungen et al. 1989). Nevertheless, the biological significance of such effects is questionable.

In the present study, external genital abnormalities as those reported by Gray and Ostby (1995) and Flaws et al. (1997) were not observed. This may be due to strain or dose differences, Also, the sexual cycle of the F_1 females was not affected, in contrast to the increased incidence of constant estrus displayed by female offspring exposed to TCDD on day 8 of gestation (Gray and Ostby, 1995). A slight but statistically significant decrease in the estrous length was reported in the lowest and middle dose groups. In the highest group the estrous length was comparable to the control. This effect seems to be an inverted U-response, but the use of multiple doses is necessary for confirmation. Nevertheless, the doses of TCDD used in this study have not impaired the initiation of pregnancy nor the reproductive outcome. Furthermore, the uterus weight was significantly reduced in the TCDD 300/60 group. The uterotrophic assay is one of the primary in vivo assays used to determine estrogenicity or antiestrogenicity of endocrine-disrupting chemicals. Chemicals with estrogen activity are known to cause a temporal advance in uterine weight, whereas antiestrogen substances inhibit the uterotrophic response elicited by exogenous estradiol (Reel et al. 1996).

In this study, exposure to low doses of TCDD throughout pregnancy and lactation elicited delay in vaginal opening and reduced uterine weight in F_1 females. These effects can be regarded as an antiestrogen effect. In the male offspring rats of the same dams exposed to the same doses of TCDD, the number of sperm per cauda epididymis and daily sperm production were reduced in all TCDD groups at puberty and at adulthood (Faqi et al. 1998a). However, in the female offspring rats of the present study, an explicit effect was observed only in the TCDD 300/60

group. Taking into consideration the number of endpoints investigated and the effects observed in both males and females of the F_1 generation, it can be concluded that the male offspring are more susceptible to TCDD than female offspring when exposure occurred throughout pregnancy and lactation.

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REFERENCES

Abraham K, Krowke R, Neubert D (1988) Pharmacokinetics and biological activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: 1. Dose-dependent tissue distribution and induction of hepatic ethoxyresorufin-o-deethylase in rats following a single injection. Arch Toxicol 62: 359-368

Buelke-Sam J, Cohen IR, Wierda D, Griffey KI, Fisher LF, Francis PC (1998) The selective estrogen receptor modulator, raloxifene: A segment II/III delivery study in rats, Reprod Toxicol 12: 271-288

Chahoud I, Krowke R, Schimmel A, Merker H-J, Neubert D (1989) Reproductive toxicity and toxicokinetics of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 1. Effects of high doses on the fertility of male rats, Arch Toxicol 66: 567-572

Colborn T, Vom Saal FS, Soto AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans, Environ Health Perspect 101: 378-384

Faqi AS, Dalsenter PR, Merker H-J, Chahoud I (1998a) Reproductive toxicity and tissue concentrations of low doses of TCDD in male offspring rats exposed throughout pregnancy and lactation. Toxicol Appl Pharmacol 150: 383-392

Faqi AS, Dalsenter PR, Merker H.-J, Chahoud I (1998b) Effects on developmental landmarks and reproductive capability of 3,3',4,4'-tetrachlorobiphenyl and 3,3',4,4',5-pentacholorobiphenyl in offspring of rats exposed during pregnancy. Hum Exp Toxicol 17: 000-000 (in press)

Flaws JA, Sommer RJ, Silbergeld EK, Peterson RE, Hirshfield AN (1997) In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces genital dysmorphogenesis in the female rat. Toxicol Appl Pharmacol 147: 351-362

Gad SG, Weil CS (1982) Statistics for Toxicologists. In: Hayes AW (ed) Principles and Methods in Toxicology. Raven Press, New York. p 273

Gray LE Jr, Kelce WR, Monosson E, Ostby JS, and Birnbaum LS (1995) Exposure to TCDD during development permanently alters reproductive function in male Long Evans rats and hamster: Reduced ejaculated sperm numbers and sex accessory gland weights in offspring with normal androgenic status. Toxicol Appl Pharmacol 131: 108-118

Gray LE Jr, Ostby JS (1995) In utero 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. Toxicol Appl Pharmacol 133: 285-294

Hagenmaier H, Wiesmüller T, Golor G, Krowke R, Helge H, Neubert D (1990) Transfer of various polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs and PCDFs) via placenta and through milk in marmoset monkey. Arch Toxicol 64: 601-615

Koch E, Thiel R, Chahoud I, Neubert, D (1995) Four generation reproductive toxicity study with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rats. I. Toxicokinetic variations in dams and offspring. Arch Toxicol 69: 217-279

Krowke R, Chahoud I, Baumann-Wilschke I, and Neubert D (1989) Pharmacokinetics and biological activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 2: Pharmacokinetics in rats using a loading-dose/maintenance-dose regime with high doses. Arch Toxicol 63 : 356-360

Li X, Weber LW, and Rozman K K (1995) Toxicokinetics of 2,3,7,8-tetrachlorodibenzo-p-dioxin in female Sprague-Dawley rats including placental and lactational transfer to fetuses and neonates. Fundam Appl Toxicol 27: 70-76

Mably TA, Moore RW, Peterson RE (1992 a) In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 1. Effects on androgenic status. Toxicol Appl Pharmacol 114: 97-107

Mably TA, Moore RW, Goy RW, Peterson RE (1992b) In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 2. Effects on sexual behavior and the regulation of luteinizing hormone secretion in adulthood. Toxicol Appl Pharmacol 114: 108-117

Mably TA, Bjerke DL, Moore RW, Gendron-Fitzpatrick A, Peterson RE (1992c) In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 3. Effects on spermatogenesis and reproductive capability. Toxicol Appl Pharmacol 114: 118-126

Reel JR, Lamb JC, Neal BH (1996) Survey and assessments of mammalian estrogen biological assays for hazard characterization. Fundam Appl Toxicol 34: 288-305

Rose JQ, Ramsy JC, Wentzler TH, Hummel RA, Gehring PJ (1976) The fate of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin following single and repeated oral doses to the rat. Toxicol Appl Pharmacol 36: 209-226

Schardein JL (1993) Hormones and hormone antagonists. In Chemically Induced Birth Defects. Dekker, New York. p 271

Theobald HM, Peterson RE (1997) In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin: Effects on development of the male and female reproductive system of the mouse. Toxicol Appl Pharmacol 145: 124- 135

Thiel R, Koch E, Ulbrich B, Chahoud I (1994) Peri- and postnatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin: effects on physiological development, reflexes, locomotor activity and learning behaviour in rats. Arch Toxicol 69: 79-86

Van den Dungen DH, Van Dieten JA, Tilders FJ, Van Rees GP, Shoemaker J (1989) Administration of a GnRH-antagonist to immature rats affects subsequently female and male pubertal development differently. Acta Endocrinol 120: 778-784