CHILDHOOD HEALTH AND DEVELOPMENT IN RELATION TO PERSISTENT ORGANOCHLORINE COMPOUNDS

Adverse effects of TCDD on mammary gland development in Long Evans rats: A two generational study.

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Introduction

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is a well-studied ligand for the aryl hydrocarbon receptor (AhR). The AhR is expressed in the epithelial portions of the mouse mammary gland during periods of rapid proliferation [1]. Absence of the AhR (Ahr-null genotype) or treatment of mammary gland explants with TCDF resulted in suppressed lobule development of the glands [1]. Recent studies have demonstrated variable effects on mammary gland development in rat offspring exposed to TCDD on day 15 of gestation [2] or during puberty [3], in addition to reported reproductive developmental alterations [4]. Oral administration of 2.5 ug/kg TCDD on 4 separate days prior to puberty in Sprague Dawley females caused a decrease in the number of terminal end buds on postnatal day (PND) 32 and significantly smaller mammary glands [3]. However, the same authors reported no significant alterations to mammary glands in 21-day-old pups exposed to 1 ug/kg TCDD on gestation day (GD) 15.

Therefore, the current studies were designed to address the extent to which TCDD can alter the developing mammary gland. These studies involved identification of the critical window of exposure. Additionally, the effect that TCDD exposure had on lactational performance and development of the mammary gland in the F_2 generation was evaluated.

Materials and Methods

TCDD (>98% purity) in acetone (1 mg/10 ml) was obtained from Radian Corp.(Austin, TX). For the preparation of dosing solution a volume of stock TCDD was added to corn oil and the acetone removed by evaporation using a Savant SpeedVac(Savant Instruments Inc., Farmingdale, NY). Following evaporation, additional corn oil was added to achieve the desired TCDD concentration. All other chemicals were from commercial sources and were of the highest purity available.

Time-pregnant Long Evans rats [gestational day 9 (day after mating =GD0)] were obtained from Charles River Breeding Laboratories (Raleigh, NC). Females were housed in plastic cages containing heat-treated pine shavings (Beta Chips, North Eastern Products Inc., Warrensburg, NY) and given food (Purina 5001 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water ad libitum.

Pregnant dams (F_0 only) were treated by oral gavage on gestational day 15 with 1.0 ug/kg TCDD in corn oil or corn oil only for controls, in a dosing volume of 5 ml/kg . Litters were standardized to 4 males and 6 females on postnatal day 4. At weaning (PND25), animals

were housed as above in unisexual groups of 2 to 3 rats/cage.

Females (F_1) from 25 litters were observed for vaginal opening and appearance of vaginal threads. Animals were weighed on days 33 and 37. Vaginal smears began the day following vaginal opening and continued until rats were 60 days old. The F_1 females (N=52) that were used in the two-generational study were mated with untreated, confirmed breeder Long Evans males (90 days old) and inspected for vaginal plugs each morning. Presence of plug was designated gestation day 0. Animals were housed in screened-bottom cages for ease of plug detection.

The critical time of TCDD exposure leading to stunted mammary gland development in Long Evans rats was examined by dosing timed-pregnant dams (N=5/treatment time) with 1 ug/kg on gestation day (GD) 15 or 20, or PND 1, 3, 5, or 10. The glands of female pups were analyzed by whole mount analysis on PND 4 and 25.

Lactational challenge was performed on postnatal day 10. Dams (8 each group) were removed from their litters of 10 pups for 3 hours, pups were weighed and the dam returned. Pups were allowed to suckle for 30 minutes, the dam was removed and pups weighed again. The suckling period began once the dam settled on the pups. The time it took for the dam to nest on the pups was also recorded.

In whole mount analyses, mammary glands were removed on PNDs 4, 25, 33, 37, 45, 68, and 110, and pressed between two slides for several hours. They were then fixed in Carnoy's solution (6:3:1, v:v:v, ethanol, chloroform, glacial acetic acid) overnight. Glands were transferred to 70% ethanol, which was removed by gradual dilution to water. The fixed glands were stained overnight in carmine alum. Glands were rinsed in water and dehydrated in increasing alcohols. The fat pads were cleared in xylene. The glands were sealed in Permount.

Results and Discussion

Treatment of Long Evans dams with 1 ug/kg TCDD on GD 15 resulted in offspring that were significantly smaller than corn oil exposed pups when evaluated on PND 33, 37, and 45. The TCDD-exposed pups also had a significant delay in time to vaginal opening (37.6 vs. 34.2 in controls) and displayed persistent vaginal threads. However, in a group of 20 animals randomly selected for vaginal smears there was no discernable difference between TCDD-exposed and control rats in their estrous patterns. These observations are consistent with similar treatment strategies in previous reports in the rat [2,5]. Decreased weight gain and delayed vaginal opening has also been reported in the hamster [6].

The mammary epithelium of TCDD-exposed and control animals was compared in whole mount analyses on PND4, 33, 37, 45, 68, and 110. Glands from PND4 females exposed to TCDD displayed significantly fewer primary branches from the nipple, delayed migration of the epithelium through the fat pad, and fewer branches and terminal buds when compared to corn oil-treated controls (Table 1). This delayed development was also evident at PND 33 and following puberty, resulting in undifferentiated terminal end buds (TEB) and spindly structures with few lateral branches or lobules. Most glands of TCDD-exposed females evaluated at PND45 retained undifferentiated TEBs, whereas few control glands had TEB remnants. Few TEB were present in PND68 glands of TCDD-exposed females and they were completely absent in control glands. The mammary epithelium of 110 day old TCDD-exposed females possessed fully differentiated ends, yet failed to fill the fat pad resulting in sparse lobule formation. These results

differ dramatically from a previous report[2] demonstrating no difference in the number of TEB, terminal ducts, or lobules in an early stage of mammary gland development (21 days-old) and a 1.3 fold increase in TEB at PND 50 in TCDD-exposed females.

Mammary glands from pups exposed to TCDD at various times during gestation or lactation (as described above) were examined on PND4 or PND25 to ascertain the critical period of development affected. At both developmental stages the only dose timing that triggered consistently underdeveloped epithelium was GD 15. Glands from a few of the animals exposed on GD 20 displayed sparse lateral branching. However, their migration through the fat pad, terminal alveolar bud formation and primary branching was similar to controls. Glands from all postnatal dosing times were also comparable to controls. This observation is consistent with the fetal mammary gland development period in which there is migration of the mammary epithelial bud into the fat pad. This transition is thought to begin on about GD 16-17[7].

To determine the severity of the delayed mammary gland development caused by TCDD exposure, a two-generational study was designed in which the offspring (F_1) of the dosed dams (N=25) were bred to confirmed breeders. They and/or their offspring (F_2) were assessed on GD14, PND 10 (lactational challenge), and at weaning. On GD 14, TCDD-exposed F_1 females (N=8 or 9 each group)carried significantly fewer fetuses than those exposed to corn oil (13.4 ± 0.7 vs. 16.6 ± 0.5, $P<0.05$). Consistent with this observation, the TCDD-exposed F_1 females had fewer live pups than controls on PND 4 (11.9 ± 0.9 vs. 14.7 ± 0.7, $P<0.05$). This observation is similar to the reduction in pup survival reported by Gray and co-workers [8]. Interestingly, there was a pup sex bias in favor of females in the TCDD-exposed F_1 group (0.42 vs. 0.51 in controls, m/f), and 47% of the F_2 litters born to TCDD-exposed dams had greater than 66% of the litter as one sex (most were females). There were 30 litters examined in the F_2 generation. This sex ratio is similar to results in a recent human cohort study in Italy [9], in which the authors reported a significant correlation of paternal TCDD concentrations in the serum to the births of daughters (ratio=0.38, m/f). In the present study, the exposure was maternal.

The lactational performance of the F_1 dams was assessed on PND10. The amount of weight gained per litter during the 30 minute suckling period was dramatically reduced in F_2 pups of TCDD-exposed dams when compared to controls (0.4 vs. 1.9 g/litter). Additionally, when mammary glands were removed from the dams at sacrifice, there was little evidence of milk in the glands. However, the amount of time it took the dams to settle on their pups was significantly increased (11 vs. 4 min., $P<0.05$). This delay to nesting indicated poor mothering behavior in the exposed dams. Finally, the mammary gland development of the F_2 pups was examined. The majority of the F_2 females of TCDD-exposed dams possessed mammary glands with either a branching or extension defect (many had both). However, the developmental defect was not as consistent as seen in the F_1 generation.

In conclusion, the persistent delay in mammary gland development caused by TCDD exposure in utero leads to a lactational defect in F1 females and to fewer and smaller pups, an event that is probably not due solely to the lactational defect. The effect of TCDD exposure is permanent and carries over into the F2 generation, adversely affecting mammary gland development.

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Table 1. *PND4 Mammary Gland Development*

	Primary Branches	% Extension to Node	Terminal Structures
Control	3.7±0.1	100	109±2.7
TCDD (1 ug/kg)	2.5±0.2*	74.6	48±8.4*

*Significantly different from corn-oil treated controls ($P<0.05$). Mean ± S.E. N=6 dams each group, 2 pups each, inguinal glands 4 and 5.