FUNCTIONAL DEVELOPMENTAL TOXICITY OF LOW DOSES OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN AND A DIOXIN-LIKE PCB (169) IN LONG EVANS RATS AND SYRIAN HAMSTERS: REPRODUCTIVE, BEHAVIORAL AND THERMOREGULATORY ALTERATIONS.

L. Earl Gray Jr., Joseph Ostby, Cynthia Wolf, Diane B. Miller, William R. Kelce, Christopher J. Gordon and Linda Birnbaum (with technical assistance of Ora Huey). Health Effects Research Laboratory, MD-72, Research Triangle Park, North Carolina, USA, 27711.

1. Introduction

2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), which alters the levels of many hormones, growth factors, hormonal receptors and hormone synthesis, is an example of an "endocrine disrupter" that acts on more than one component of the endocrine axis at a time[1]. As a consequence, TCDD adversely affects reproduction and development in several vertebrate classes, including mammals. Furthermore, it has been demonstrated that the embryo is extremely sensitive to 2,3,7,8 TCDD and related chemicals, with adverse developmental effects occurring at dosage levels two or three orders of magnitude below those required to alter reproductive function in adult animals. For example, offspring of Wistar rats display reduced fertility after administration of 0.5 μg TCDD/kg/d from gestational day (GD) 6 to 15[2]. In a multigeneration study[3], fertility was reduced in Sprague-Dawley rats in the F1 and F2 at 0.01 μg TCDD/kg/d in the diet. Mably et al.[4,5,6] reported that a single exposure to TCDD at dosage level as low as 0.064 μg/kg on GD 15 alters sexual differentiation of male Holtzman rat progeny (dose levels ranging from 0.064 to 1 μg TCDD/kg). Subsequent studies[7,8,9,10,11] using both Holtzman and LE rats, exposed to 0.7 to 1.0 μg TCDD/kg on GD 15, have replicated some, but not all, of the reproductive alterations reported by Mably et al[4,5,6]. However, those effects that do replicate are generally less robust than initially reported at comparable dosage levels. Along with TCDD, PCB/PCDF exposures are also clearly linked to developmental/reproductive toxicity in humans, primates, rodents, mink, fish and other wildlife species. For example, administration of PCB congener 169 on GD 1 at 1.8 mg/kg reduced fertility in both male and female offspring[12].

2.OBJECTIVES

Seven research objectives are addressed in this series of studies. Our objectives were to expand the dose-response observations of Mably et al[4,5,6] on the developmental effects resulting from administration of TCDD on a single day of gestation to, 1) another strain of rat, 2) another species, the hamster[8], 3) an earlier stage of gestation[8], and 4) the female offspring[13]. We also examined 5) neurobehavioral (male rat) and 6) thermoregulatory[14] (male rat and hamster) function in offspring during adulthood after gestational TCDD administration. In a final study, we examined 7) the effects of in utero

exposure to the TCDD-like PCB congener # 169 on the reproductive system of male and female LE rat offspring through 70 days of age[15].

3.METHODS

LE rats were dosed by gavage with 0, 0.05, 0.20, 0.80 or 1 μg TCDD/kg in oil on GD 15 or 1 μg TCDD/kg on GD 8. Holtzman rats were also dosed with 1 μg TCDD/kg on GD 15. In addition, pregnant Syrian hamsters, a species relatively insensitive to the lethal effects of TCDD, were dosed on GD 11, developmentally equivalent to GD 15 in the rat, with TCDD at 2 μg/kg. In a final study, PCB 169 was administered at 1.8 mg/kg on GD 8 to pregnant LE rats. In these studies, reproductive organ weights, testicular, epididymal and ejaculated sperm counts, testosterone levels, estrous cyclicity, reproductive behavior, fertility and histology were assessed in the offspring.

Neurobehavioral testing was conducted on middle-aged adult (1 μg TCDD/kg on GD-15 and control) male offspring. Female offspring were not tested. In this effort, a variety of behavioral endpoints were assessed to determine if TCDD exposure altered sexually dimorphic nonreproductive behavior (exploratory and residential activities, saccharin preference) or behavior known to be affected by hypothyroidism (Morris water maze).

1) FIGURE-8 MAZE ACTIVITY. Vertical (locomotor) and horizontal (rearing) activities (exploratory and residential) were assessed in Figure-8 mazes. Rats resided in the mazes for a period of one week. Data were recorded in 10 min intervals for the first two hours (exploratory activity) and in one hour intervals thereafter (residential).

2) HOME CAGE ACTIVITY AND 3) SACCHARIN PREFERENCE. Activity was also evaluated in the home cage (46 cm x 25 x 15) utilizing an automated system. Interruption of any one of the 3 infrared photobeams was recorded as an index of activity. Activity data were collected in one hour intervals for 20 days. At this time, animals were presented with a choice of tap water or saccharin solution, provided in ascending order of saccharin concentration (0 versus 0.06, 0.125, 0.25, 0.50, 0.75 %) for three days per trial, over a 20 day period. Tap water was present in both bottles on nonchoice days.

4) MORRIS WATER MAZE. This apparatus is a circular (1.83 m diameter, 0.58 m deep) pool filled with water (27°C) made opaque by the addition of nontoxic white paint. A white escape platform (height 34.5 cm) was located 1 cm below the surface near the center of one of four quadrants. Rats were given two trials (1 hour apart) per day until latency to reach the platform declined to 10 sec or less. Attainment of the criterion was followed by a probe test (90 sec) in which the platform was unavailable.

The effects of perinatal exposure to TCDD on metabolism and temperature regulation were assessed in adult male rat and hamster offspring. To this end, individual male rat offspring were placed in a gradient-layer calorimeter for 5 hr during their nocturnal period while ambient temperature (T_a) was maintained at 10, 16, 24, or 28 °C. Metabolic rate, as measured from the total heat loss in the calorimeter was determined along with evaporative heat loss, dry thermal conductance, and body core temperature (T_c). Male hamster offspring were tested in a similar manner over over a T_a range of 14 to 34 °C.

4. RESULTS

4.A.1 REPRODUCTIVE EFFECTS OF TCDD IN MALE OFFSPRING[8]

When LE rat dams were dosed with 0, 0.05, 0.20, 0.80 or 1.0 μg TCDD/kg on GD 15, growth and viability of the pups were reduced at 0.80 and 1.0 μg TCDD/kg. The onset of eyeopening was accelerated in all treated groups, including 0.05 μg TCDD/kg. Puberty (the

age at preputial separation) was delayed in male offspring from the 0.20 and 0.80 μg TCDD/kg dosage groups. Treatment with 1 μg TCDD/kg delayed puberty by 3.6 days after GD-15 dosing and by 2.1 days after GD-8 administration. In 49 day old male progeny, ventral prostate and seminal vesicle weights and epididymal sperm counts were reduced by treatment with 0.80 μg TCDD/kg on GD-15. At 63 days of age, GD 15 TCDD administration reduced epididymal sperm numbers in male offspring by 10 % in the 0.20 μg/kg group and 25 % in the 0.80 μg TCDD/kg dosage group. Cauda epididymal weight was also reduced by about 15 % by GD 15 treatment with 0.80 μg TCDD/kg. Currently, data on the LE rat after 70 days of age are available for only the 1.0 μg TCDD/kg and the concurrent control groups.

At middle age, male offspring from the 1 μg TCDD/kg-GD 15 treatment group had 38 % fewer epididymal sperm, 20 % less testicular sperm and 58 % fewer ejaculated sperm numbers than did control male rats. The numbers of copulatory plugs found after mating were not reduced by GD 15 or GD 8 treatment with 1 μg TCDD/kg. Hence, the numbers of ejaculated sperm per plug were reduced by 49 % ($p < 0.005$) in the GD 15 (15.9×10^6/plug) and 20 % in the GD 8 (24.8×10^6/plug) 1 μg TCDD/kg exposure groups versus control (31.1×10^6/plug), indicating that the reduced numbers of ejaculated sperm in the uterus of the females, paired with TCDD-treated male offspring, did not result from a lack of mating or an inability to ejaculate. In addition, treatment on GD 15 at 1μg TCDD/kg permanently reduced testis, seminal vesicle and cauda epididymal weights. Although testis weights were altered, histopathological alterations were in a small percentage of middle-aged GD 15 males, which displayed atrophic testes.

In male LE rat offspring exposed to 1 μg TCDD/kg, we observed alterations of male sexual behavior as a result of GD 15 treatment. The total number of mounts, with and without intromission, was doubled as a consequence of perinatal exposure to 1 μg TCDD/kg and the latency to ejaculate was increased. However, 1 μg TCDD/kg-exposed male offspring were not feminized as all groups displayed similar levels of feminine sexual behavior after castration and treatment with female sex hormones. In addition, the quality of the lordosis response, when it occurred, did not differ between the groups.

In the males necropsied on PND 0, 49, 63, and adulthood, androgenic measures (i.e., serum testosterone and LH-stimulated *in vitro* testosterone production (PND 0, 49, adulthood)), were not reduced by administration of 1 μg TCDD/kg on GD 15. Interestingly, many testosterone-dependent processes, including male sex behavior, anogenital distance, age at puberty and sex accessory gland weight, are reduced in spite of the fact that these androgenic measures were unaffected in TCDD treated male offspring at any age.

In the Syrian hamster, administration on GD 11 (day 0 is day after mating) with 2 μg TCDD/kg demasculinized male Syrian hamster offspring. In this species, puberty (preputial separation) was delayed by about 3 days and reductions were noted in ejaculated and epididymal sperm numbers and seminal vesicle weight. Similar to rat offspring, testicular sperm production was much less affected than epididymal or ejaculated sperm numbers and serum testosterone was not reduced in TCDD exposed male hamster offspring. Some reproductive measures, such as anogenital distance and male sex behavior were altered by TCDD-treatment in rat but not hamster offspring.

REPRODUCTIVE EFFECTS OF TCDD IN FEMALE OFFSPRING[13]

Treatment with TCDD at 1 μg/kg on GD-15 delayed puberty and induced a number of unusual reproductive alterations. More than 65 % of the female offspring displayed complete to partial clefting of the phallus, and 80 % displayed a permanent "thread" of tissue across the opening of the vagina as a consequence of treatment with 1 μg TCDD/kg on GD-15. In the GD-8 treatment group, 25 % displayed partially cleft phallus and 14 % had a vaginal "thread".

GD-15 TCDD administration also induced a high incidence of malformations in Holtzman female progeny (100 % clefting and 83 % with a vaginal thread). At lower doses, puberty was delayed by treatment with 0.80 μg TCDD/kg on GD-15 and treated females displayed reproductive malformations, including persistent vaginal thread (33% at 0.20 and 66% at 0.80 μg TCDD/kg) and partial to complete clefting of the phallus (10 % at 0.20 μg TCDD/kg and 60% at 0.80 μg TCDD/kg).

In young adult LE female offspring, vaginal and behavioral estrous cyclicity, estrous cycle-mediated running wheel activity and female sexual behaviors at proestrus (darting and lordosis to mount ratios) were not affected by treatment on GD-15 with 1 μg TCDD/kg. However, untreated males had difficulty attaining intromission, took longer to ejaculate and induced vaginal bleeding when mated with GD-15 TCDD-exposed female offspring. Although GD-8 administration of 1 μg TCDD/kg produced a lower levels of developmental toxicity and malformations than did GD 15 treatment, the female offspring from the GD 8 treatment group displayed enhanced incidences of constant estrus (CE) (47 % CE in GD-8 versus 16 % CE in the control and GD-15 groups at middle age) and cystic endometrial hyperplasia. In addition, in the GD-8 group the fertility rate in continuous breeding conditions declined significantly faster than in control females ($p < 0.02$). Only 19 % of GD-8 female progeny producing a fifth litter versus 61 % in the control group ($p < 0.012$). Overall fecundity was reduced by 38 % by GD 8 treatment with 1 μg TCDD/kg ($p < 0.07$). When middle aged female offspring were necropsied, ovarian weight was reduced by 23 % in both rat strains by GD 15 treatment and by about 30 % by GD 8 treatment with 1 μg/kg TCDD.

Female hamster offspring were seriously affected by in utero exposure to 2 μg TCDD/kg, with 100 % displaying clitorine clefting and reduced fecundity, measured from a single fertilty trial. was dramatically reduced. In treated female hamster offspring, vaginal opening was delayed and vaginal estrous cycles were abnormal, but behavioral estrous cyclicity was generally unaffected. Most treated female offspring mated and became pseudopregnant or pregnant, but typically the F2 pups were either born dead, or died before weaning. Taken together. Our results demonstrate that of a administration of TCDD late in gestation results in similar malformations of female external genitalia in two species of rodents.

4.A.2 TCDD-NEUROBEHAVIORAL TEST RESULTS: ADULT LE HOODED MALE RATS

In summary, although TCDD treatment altered male rat sex behaviors, no significant alterations were noted in any of the above neurobehavioral tests. However, behavior should be reassessed in younger animals to determine if neurobehavioral development is delayed, or altered in infantile, pubertal or young adult animals.

4.A.3 EFFECTS OF TCDD ON TEMPERATURE REGULATION AND METABOLISM IN ADULT LE RATS AND SYRIAN HAMSTERS[14]

Rats exposed to TCDD in utero had a significantly lower body temperature at T_as of 10, 16 and 24 degrees C and a higher thermal conductance. Metabolic rate was unaffected by TCDD, indicating, that TCDD did not impair the regulation of T_c during cold exposure. Evaporative heat loss was also unaffected by TCDD. Skin blood flow of the interscapular area was measured in anesthetized rats with laser Doppler velocimetry and found to be the same in control and TCDD groups. Adult male hamster offspring, exposed to TCDD in utero were also significantly hypothermic over a T_a range of 14 to 34 °C. As in the rat, metabolic rate was unaffected, whereas thermal conductance was significantly elevated. The reduction in body temperature over a wide range of ambient temperatures, concomitant with normal thermoregulatory effector function, suggests that perinatal TCDD exposure results in a reduction in the regulated body temperature (i.e., decrease in set-point).

4.B REPRODUCTIVE EFFECTS OF PCB 169[15]

GD 8 administration of 1.8 mg PCB-169/kg produced TCDD-like alterations of reproductive development in male and female rat offspring. In the PCB 169 treatment group, growth was retarded while eyeopening was accelerated. In treated male offspring, puberty was delayed, and at 65 days of age, ventral prostate and seminal vesicle weights and cauda epididymal sperm counts were reduced by at least 50 % of the control value. In contrast, testis spermatid counts were only reduced by about 20 % of the control mean. In treated female offspring, about 50% displayed a permanent vaginal thread and malformations of the external genitalia.

5. DISCUSSION

We have found that in utero administration of TCDD results in profound disruption of reproductive, behavioral and thermoregulatory function in male and female offspring[13,14,15,16] in addition to reducing postnatal viability and growth. Such effects were seen in two rodent species, the hamster and the rat, and two rat strains, at dosage levels that fail to induce overt maternal toxicity. In rat offspring, postnatal mortality in rats occurred at 1.0 μg/kg, growth was retarded at 0.80 μg/kg, reproductive malformations and delayed puberty were noted at 0.20 μg/kg and eyeopening was accelerated at 0.05 μg TCDD/kg.

TCDD-exposed female hamster offspring display malformations of the external genitalia, subfertility and abnormal vaginal opening and cyclicity. Female rat offspring, exposed to either TCDD or PCB 169 display malformed external genitalia, and a persistent vaginal thread after puberty. In addition, GD-8 TCDD-treated female rat offspring are subfertile, they enter constant estrus at an early age and display a higher incidence of mild cystic endometrial hyperplasia. In addition, one TCDD-treated female rat also displayed a rare form of uterine cancer, an observation that warrants replication. Male rat and hamster offspring display consistent and reproducible reductions in epididymal and ejaculated sperm numbers and sex accessory gland weights. Since serum testosterone (T), T production by the testis in vitro, and androgen receptor numbers in sex accessory glands and the epididymis are not altered by in utero TCDD exposure, the size reduction in these tissues are not likely to have resulted from an alteration of the androgenic status of the male offspring. Results of our study using Holtzman rats are nearly identical to the effects seen in the LE rat. Hence, we were able to replicate some, but not all of the effects of TCDD on male Holtzman rat progeny, reported by Mably et al.[4,5,6]. In particular, our results differ from those reported by Mably et al.[4,5,6] who observed alterations in many of the above endpoints at lower dosage levels (i.e., epididymal sperm counts were reduced by about 50 % at 0.064 μg/kg in 63 day old males). In our LE rat dose-response study, epididymal sperm counts are reduced by about 10 % in the 0.20 and 25 % in the 0.80 μg TCDD/kg dosage groups (day 63), respectively. Similar to the effects of TCDD, treatment with PCB 169 early in gestation produces a 50 % reduction in epididymal sperm numbers and ventral prostate and seminal vesicle weights in 63 day old male offspring.

Adminstration of a dioxin-like PCB (169) on GD 8 produced TCDD-like alterations of growth and reproductive anatomy and function in LE male and female rat offspring. Hence, the novel pattern of developmental alterations that results from in utero administration of TCDD is not unique to TCDD, but rather, it may be pathognomonic for the developmental effects of Ah-receptor mediated toxicants in general. In conclusion, TCDD and a TCDD-like PCB congener are potent developmental reproductive toxicants at relatively low dosage levels. However, the functional developmental toxicity that results from in utero TCDD exposure is not limited to the reproductive system. Novel alterations of thermoregulatory function were detected in mature rats and hamsters as a consequence of in utero TCDD treatment.

REFERENCES

1) Birnbaum, L. S. (1994). Endocrine effects of prenatal exposure to PCBs, Dioxins, and other Xenobiotics: Implications for Policy and Research. Environ. Health Perspectives 102, 676-679.
2) Khera, K.S. and Ruddick, J.A. (1973). Polychlorodibenzo-p-dioxins: Perinatal effects and the dominant lethal test in Wistar rats. In: Chlorodioxins-origin and fate (E.H. Blair, Ed.) pp. 70-84. American. Chem. Soc., Washington, D.C.
3) Murray, F.J., Smith, F.A., Nitschke, K.D., Humiston, C.G., Kociba, R.J., and Schwetz, B.A. (1979). Three-generation reproduction study in rats given 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the diet. Toxicol Appl Pharmacol 50, 241-252.
4) Mably T., Moore. R.W., Peterson, R.E. (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.
5) Mably, T., Moore, R.W., Goy, R.W, Peterson, R.E. (1992 b). 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.
6) Mably, T., Bjerke, D.L., Moore, R.W., Gendron-Fitzpatrick, A., Peterson, R.E. (1992 c). 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.
7) Chen, S.W., Roman, B.L., Saroya, S., Shinomiya, K., Moore, R.W., Peterson, R.E. (1993). In utero exposure to 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) does not impair testosterone production by fetal rat testes. The Toxicologist 13, 104.
8) Gray, L.E., Kelce, W.R., Monosson, E., Ostby, J.S., and Birnbaum, L.S. (1995). Exposure to TCDD during development permanently alters reproductive function in male LE rats and Hamsters: Reduced ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal androgenic status. Toxicol and Appl Pharmacol 131, 108-118.
9) Wilder, C.E., Welsh, T.H. Jr., and Johnson, L. (1995). Effects of perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on sertoli cell number and testicular function. The Toxicologist 15, 293.
10) Bjerke, D.L., Brown, T.J., MacLusky, N.J, Hochberg, R.B., and Peterson, R. E. (1994). Partial demasculinization and feminization of sex behavior in male rats by in utero and lactational exposure of male rats to 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) is not associated with alterations in estrogen receptor binding or volumes of sexually dimorphic brain nuclei. Toxicol Appl Pharm 127, 258-267.
11) Klinefelter, G. R., Boman, B.L., and Petersen, R.E. (1995). A single gestational TCDD exposure alters protein expression in the adult rat epididymis. The Toxicologist 15, 233-234.
12) Smits-van Prooije, A.E., Lammers, J, Waalkens-Berendsen, D.H., Kulig, B.M. and Snoeij, N.J. Effects of the PCB 3,4,5,3',4',5' hexachlorobiphenyl on the reproduction capacity of Wistar rats. Chemosphere 27, 395-400.
13) Gray, L.E. Jr., and Ostby, J.,S.(in press). In utero 2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD) Alters Reproductive Morphology and Function in Female Rat Offspring. Toxicol Appl Pharm.
14) Gordon, C.,J., Gray, L.E.,Jr., Monteiro-Riviere, N.,A. and Miller,D.B. (in press). Temperature regulation and metabolism in rats exposed perinatally to dioxin: Permanent change in regulated body temperature. Toxicol. Appl. Pharm.
15) L.E., Gray Jr. and W.R.Kelce. (submitted) Latent effects of pesticides and toxic substances on sexual differentiation of rodents. Toxicol Indust Health.