2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) disrupts early morphogenetic events that form the lower reproductive tract in female rat fetuses

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Introduction

Exposure to TCDD and dioxin-like compounds results in a wide variety of effects in experimental animals, including a wasting syndrome, immunosuppression, thymic atrophy, chloracne, teratogenicity, carcinogenicity, as well as other toxic and biochemical effects [1]. In addition, TCDD exposure induces reproductive and developmental effects in experimental animals and wildlife. In rats, *in utero* exposure during critical periods of organogenesis causes a wide range of adverse effects, including delayed puberty and reduced sperm counts in males and an increased incidence of cystic endometrial hyperplasia, constant estrous, and reduced fecundity in females [2-4].

A single oral dose of 1.0 μg TCDD/kg on gestation day (GD) 8 or GD15 caused structural and functional abnormalities in the female rat reproductive system, including reduced fecundity and the presence of a vaginal thread [3, 4]. In the study by Gray and Ostby, no distinction was made between whether the vaginal thread was due to gestational exposure to TCDD, which altered normal embryogenesis, or whether TCDD interferes with normal vaginal opening. Flaws and coworkers showed that the vaginal thread in TCDD-exposed rats consists of mesenchyme surrounded by epithelial cells and was clearly visible in histological sections as early as PND2 [5]. This indicates that the mechanism responsible for occurrence of the vaginal thread would appear to involve earlier events, rather than effects at vaginal opening.

The objective of this study was to investigate TCDD-induced alterations during embryogenesis to better understand vaginal thread formation. In this study, time-pregnant Long Evans rats received a single, oral dose of 1.0 μg TCDD/kg on GD15. Dams were sacrificed on GD17, 18, 19, and 21 and the reproductive tracts from female fetuses were examined histologically to determine TCDD's effect on vaginal development. Results indicate that abnormalities were detected as early as GD18 in Mullerian duct fusion (a process critical to vaginal morphogenesis) and these effects constitute the origin of the vaginal thread. Subtle differences were also present as early as GD17. This information may help elucidate the mechanism of action of TCDD-induced effects on vaginal development in the rat.

Materials and Methods

Treatment of Animals

Female, time-pregnant, Long Evans rats (8-12 weeks old, ~225g) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Rats were dosed by oral gavage on gestation day (GD) 15 with 1.0 μg TCDD/kg in 5 ml corn oil/kg (n=3-5). Dams were sacrificed on GD17-21 and individual reproductive tracts were isolated from female fetuses.

Histology

Female rat reproductive tracts were stained using a Feulgen nuclear staining protocol as described by Whiting [6]. The stained tissues were embedded in paraffin and sectioned to examine gross morphology, presence of mitotic figures, and localized patterns of cell death.

Microscopic Analysis

All sections (20 or 30 μm thickness) were examined under a Leitz Laborlux D microscope (25x). For each gestation day, distinct morphological features, referred to as "landmarks," were identified in the caudal to cranial axis of the lower reproductive tract. The following landmarks include the first appearance of individual Mullerian ducts, fusion of Mullerian ducts, and appearance of Wolffian ducts. Of particular interest was progression of Mullerian duct fusion in the lower reproductive tract. This was assessed by measuring the distance spanned by unfused Mullerian ducts as well as the determination of the degree of separation of the left and right Mullerian ducts, which was measured as the width of the intervening mesenchyme.

Using serial sections, the distance between each of the major morphological landmarks was determined progressing from a caudal to cranial direction. Measurements of mesenchyme thickness were determined in the section in which the Mullerian ducts were viewed as two distinct structures.

Statistical Analysis

Comparisons of mesenchyme thickness between the Mullerian ducts in control and TCDD-treated samples were analyzed by t-tests (SigmaStat, Jandel Corporation). Differences between treatment groups were considered significantly different when $p < 0.05$. All data are presented as means ± standard deviation.

Results and Discussion

The goal of the present study was to compare the effects of TCDD treatment on vaginal development in prenatally exposed rats. During normal development, it is thought that the vagina is formed from two embryonic regions [7, 8]. The lower two-fifths of the vagina are formed from the endodermally derived urogenital sinus, while the upper three-fifths of the vagina from the mesodermally derived Mullerian ducts (MDs). The development of the genital tract goes through an indifferent stage in which both male and female structures are present [9]. In female fetuses, the absence of testosterone causes the mesonephric tubules and Wolffian ducts (WDs) to regress [10].

On GD17, there was no difference in the width of the mesenchyme between the MDs in TCDD-treated fetuses. However, there were subtle differences in the position and fusion of the MDs. Progressing in a caudal to cranial direction, the MDs in control fetuses were uniformly separated in the unfused zone and fusion occurred within 275 μm (Fig. 1A). However, in fetuses exposed to TCDD, the orientation of the MDs was somewhat different. The spacing of the MDs within the mesenchymal tissue was such that a profile of the ducts was bell-shaped and MD fusion occurred within a shorter distance (~240 μm) (Fig. 1B). Although it is not clear how these changes are related to the vaginal thread formation, they do indicate that within 48 hrs of treatment, TCDD elicits subtle morphological changes within the developing reproductive tract. Measurements of the separation of the MDs indicated no statistically significant difference in mesenchyme width as this stage of gestation.

Prenatal exposure to TCDD produced distinct abnormalities in the female reproductive tract as early as GD18. For example, the length of the vaginal tract from the time it separated from the urethra to the first appearance of the Mullerian and Wolffian ducts was considerably shorter in

exposed pups (Fig. 2). However, the length of unfused MDs was the same in both treatment groups (Fig. 2). Although the relative length in which MD fusion occurred did not appear to be affected, the distance (caudal to cranial) between morphological features was significantly altered. For example, in TCDD-treated fetuses the distance from landmark 2 to landmark 3 was significantly greater (Fig. 2). Although this is not the region of MD fusion, TCDD affected the relative positioning of distinguishable landmarks within the developing reproductive tract. On GD19 and GD21 the length of unfused MD was significantly greater in TCDD-treated fetuses than in controls. On GD19, the length of unfused ducts in TCDD-treated fetuses was 68 μm (35% of length) compared to 38 μm (13%) in control (Fig. 3). Similar patterns were seen on GD21: 67 μm (4.7%) in control fetuses versus 315 μm (23%) in exposed (Fig. 4).

As early as GD18, the width of mesenchyme separating the MDs was greater in fetuses exposed to TCDD versus control fetuses (Table 1). Although the mesenchyme width decreased in control and treated reproductive tracts over time (GD18, 19 and 21), TCDD-exposed fetuses had significantly more mesenchyme between the MDs (Table 1). The vaginal thread may, in fact, be the retained zone of unfused MDs, including the mesenchymal core described here. During normal MD fusion, mesenchyme removal may involve migration of these cells out of the zone between the left and right MDs. Processes involved in disruption of fusion could include failure to migrate out of the zone, stimuli that induce proliferation or recruit cells into the zone, and potential disruption of programmed cell death in the interductal zone. Growth factor expression as well as synthesis and remodeling of extracellular matrix are potential regulators of these processes and members of the TGF-β family are known to regulate such events. There is also evidence that TCDD disrupts growth factor expression in embryonic urinary tract affecting epithelial cell proliferation and matrix protein expression [11, 12]. The TGF-βs are growth inhibitory to epithelial cells but can stimulate or inhibit proliferation of mesenchymal cells depending on the entire set of expressed growth factors [13]. These peptides may be implicated in the pathology of vaginal thread formation. (This abstract does not necessarily represent EPA policy. CHH supported by EPA, CT 902908).

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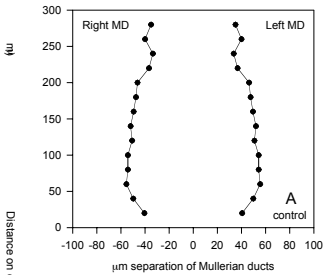

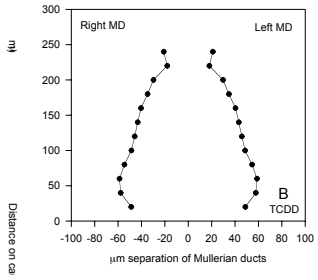

Figures 1A and 1B. Distances on the cranial-caudal axis between specific morphological features. Measurements of Mullerian duct separation were made from the section in which two distinct Mullerian ducts were present until Mullerian duct fusion. Values represent the mean of 4-5 fetuses.

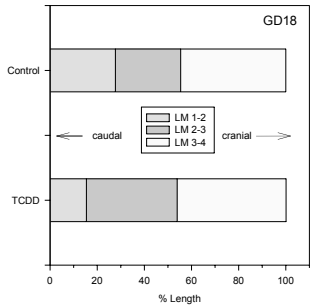

Fig. 2

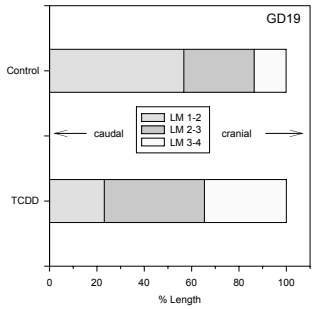

Fig. 3

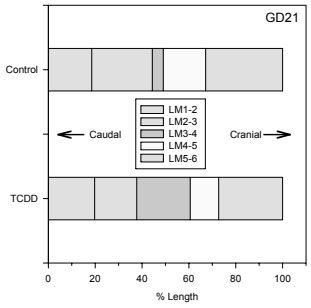

Fig. 4

Figures 2 and 3. Relative length between specific morphological features on the caudal-cranial axis on GD18 and GD19. Landmark (LM) 1: urethra-vaginal separation. LM2: Separation of left and right Mullerian ducts (MDs). LM3: Appearance of distinct MDs and Wolffian ducts. LM4: MD fusion.

Figure 4. Relative length between specific morphological features on the caudal-cranial axis on GD21. Landmark (LM) 1: urethra and surrounding mesenchyme appear "bell-shaped". LM2: urethra-vaginal separation. LM3: appearance of left and right Mullerian ducts. LM4: Mullerian duct fusion. LM5: oval vaginal lumen, which is filled with epithelial plug of cells. LM6: oval vaginal lumen, but epithelial plug has been removed.

Table 1

Distance between left and right Mullerian duct (μm)

Gestation Day	Control	TCDD
18	111.0 ± 10.5	138.0 ± 8.5*
19	79.5 ± 5.0	117.0 ± 18.9*
21	26.5 ± 12.1	79.5 ± 24.9*

Values expressed are mean ± standard deviation

* Significantly different from control ($p<0.05$)

Measurements of the distance between the two MDs were taken at the first instance of both MDs being present.