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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Interacts with Endogenous Estradiol to Disrupt Prostate Gland Morphogenesis in Male Rat Fetuses

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Fetal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) interferes with normal development of the male reproductive system in rats and mice. We examined the effects of TCDD on the initial development of the urogenital system (urethra, prostate, and seminal vesicles) in male rat fetuses on gestation day (GD) 20. The number of prostatic buds and size of prostate glands as well as seminal vesicle size was determined by computer-assisted 3D reconstruction. Pregnant Holtzman rats received a single oral dose of TCDD (1 μg/kg) on GD 15. The intrauterine position (IUP) of male fetuses was identified based on the sex of adjacent fetuses: 2F males were located between 2 females and 2M males were located between 2 males. Control 2F males had elevated serum estradiol and larger prostates than control 2M males, which had elevated serum testosterone and larger seminal vesicles, confirming prior findings. There was no effect of TCDD on serum testosterone. TCDD significantly decreased the number of buds in the dorsocranial and dorsolateral regions of the urogenital sinus and overall prostate size, and was associated with a significant decrease in serum estradiol only in 2F males. In contrast, in 2M males both serum estradiol and the number and size of prostatic buds in these same regions of the prostate were unaffected by TCDD, although seminal vesicle size was reduced. These findings show that individual differences in gonadal steroid levels influence the response of the developing prostate to TCDD in male fetuses. In addition, these TCDD effects may be mediated in part by a decrease in serum estradiol levels.

Key Words: **2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); prostate development; intrauterine position; testosterone; estrogen; rat.**

The toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are now well known from animal studies, although the range of effects of dioxin on human health are not as well understood (Brouwer *et al.*, 1998; Grassman *et al.*, 1998; Peterson *et al.*, 1992). Previous work on the effects of TCDD in Holtzman rats revealed that a single administration of a low dose of TCDD on gestation day (GD) 15 resulted in a reduction in adult ventral prostate weight (Mably *et al.*, 1992; Roman *et al.*, 1995; Roman and Peterson, 1998). In addition, impairment of growth of the fetal prostate was also reported (Roman *et al.*, 1998). These effects were not associated with a change in circulating androgen, suggesting a direct effect of TCDD on the prostate rather than an indirect effect via alteration in the secretion of testosterone (Roman *et al.*, 1995), or conversion of testosterone to 5α-dihydrotestosterone (DHT; Theobald *et al.*, 2000a,b).

The prostate in male rats begins developing on GD 18.5 (Timms *et al.*, 1994). Induction of prostatic budding appears to be regulated by the underlying urogenital sinus (UGS) mesenchyme (Hayward *et al.*, 1997). Epithelial buds develop bilaterally in specific anatomical regions of the UGS: dorsocranial, dorsal, lateral, and ventral (Fig. 1). By GD 20 (the time at which we examined male fetuses in this study), the initial budding process is complete. Subsequent epithelial glandular growth and branching continues postnatally (Hayashi *et al.*, 1991; Prins and Birch, 1997).

Testicular secretion of testosterone begins on GD 15 in rats (Warren *et al.*, 1973) and is a requirement for differentiation of the reproductive organs that develop from the UGS (prostate and other glands), Wolffian ducts (epididymides, vas deferens, and seminal vesicles), and perineal tissue (penis and scrotum; vom Saal *et al.*, 1992). In addition, normal development of the UGS and external genitalia require expression of the enzyme 5α-reductase that converts testosterone to DHT. Androgen receptors are abundant in the UGS mesenchyme (Cooke *et al.*, 1991; Prins and Birch, 1995; Timms *et al.*, 1999), and binding of DHT to androgen receptors in mesenchyme is required for epithelial differentiation (Cunha *et al.*, 1987). While expression of androgen receptors in epithelium is not required for the initial budding and growth of epithelium (Cunha *et al.*, 1987), there is, nonetheless, a low level of androgen receptor (Timms *et al.*, 1999) as well as estrogen receptor gene expression (Prins *et al.*, 1998) in the epithelium during the initial period of prostate gland genesis.

Timms and colleagues have described a 3-D reconstruction

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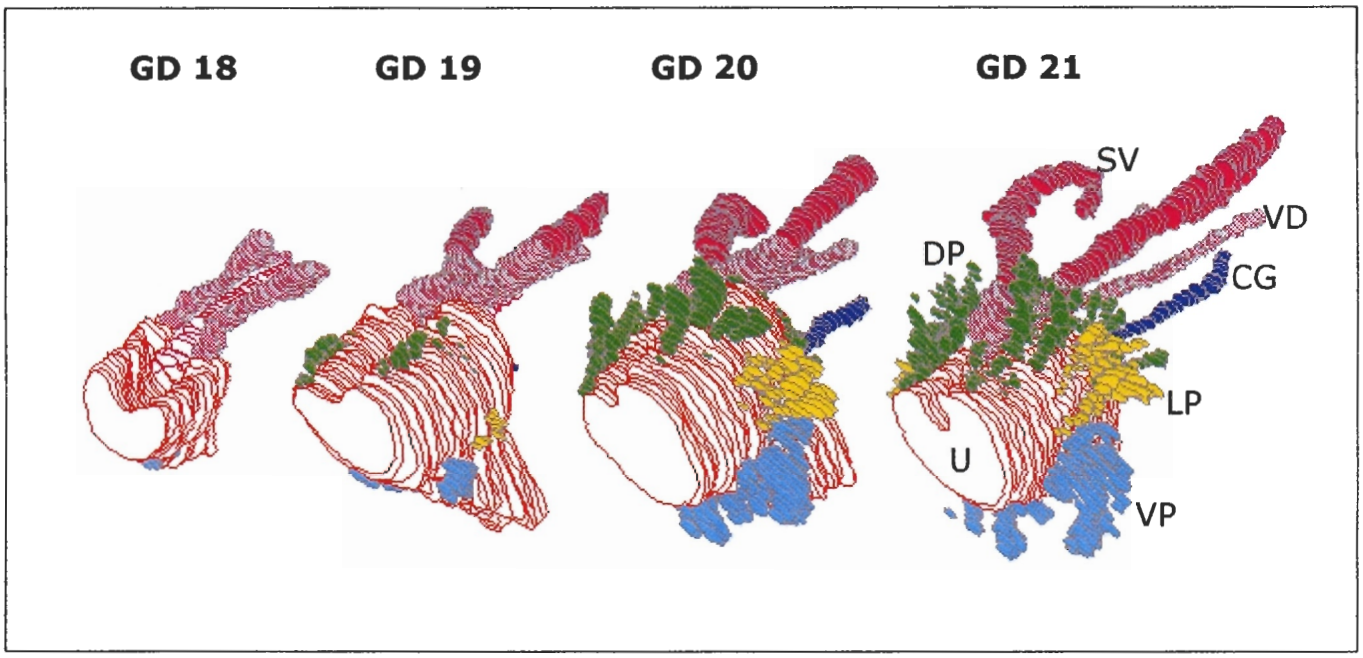

FIG. 1. Serial section reconstructions of the rat urogenital complex on GD 18, 19, 20 and 21, illustrating the stages of early prostate development. Prostate morphogenesis begins at GD 18 with small ventral prostate buds and by GD 19 the dorsal, ventral, lateral, and coagulating gland buds are visible. On GD 21 (the day before birth), prostate budding from the UGS is essentially complete. U, urethra; DP, dorsal prostate; LP, lateral prostate; VP, ventral prostate; CG, coagulating gland; SV, seminal vesicle; VD, vas deferens.

technique for studying fetal development of the prostate (Timms *et al.,* 1994). Using this technique, the development of the prostate was found to differ in male Sprague-Dawley rat fetuses that occupied different intrauterine positions (IUPs). Male fetuses that occupied an IUP between female fetuses (2F males) had a greater mean area of prostatic buds in the dorsocranial, dorsal, and lateral regions relative to male fetuses located between other male fetuses (2M males; Timms *et al.,* 1999). This finding was consistent with prior studies in mice in which as adults, 2F male mice were found to have larger prostates relative to 2M males (Nonneman *et al.,* 1992). The enlarged prostate in 2F males was hypothesized to be mediated by an elevated level of serum estradiol in 2F male fetuses relative to 2M fetuses, due to transport of estradiol from adjacent female fetuses (Even *et al.,* 1992; vom Saal, 1989). This hypothesis was confirmed in a study in which estradiol was experimentally elevated by 50% in male mouse fetuses (via maternal administration), and the estrogen-treated males showed both a significant increase in prostatic glandular buds and significantly larger buds during fetal life, as well as enlarged prostates in adulthood (vom Saal *et al.,* 1997).

Numerous studies have now shown that a small increase in estrogenic activity, either from estradiol, estrogenic drugs, or environmental estrogens in plastic or pesticides, in male mouse fetuses results in a permanent increase in prostate size during postnatal life (Gupta, 2000a,b; Nagel *et al.,* 1997; Nonneman *et al.,* 1992; Thayer *et al.,* 2001; vom Saal *et al.,* 1997; Welshons *et al.,* 1999). The increase in prostate size is associated with an increase in prostatic androgen receptors (Gupta, 2000a; Nonneman *et al.,* 1992; Thayer *et al.,* 2001; vom Saal *et al.,* 1997). It thus appears that a small increase in estrogen during the initial period of prostate development in fetal life results in an increase in the response of the prostate to androgen. In contrast, supraphysiological doses of estradiol or estrogenic chemicals can have the opposite effect of low doses and dramatically interfere with normal development of the prostate (Gupta, 2000a; Prins, 1997; vom Saal *et al.,* 1997).

In the present study we examined the effect of TCDD administration to pregnant rats on early development of the prostate in male rat fetuses, with attention being paid to the IUP of the males. The objective was to determine whether the intrauterine position of male fetuses, which is related to background levels of estradiol (elevated in 2F males) and testosterone (elevated in 2M males), would influence the response of the developing prostate to TCDD. TCDD has been shown to inhibit estrogen-induced responses in several tissues (Buchanan *et al.,* 2000; Peterson *et al.,* 1992, 1993). We report here that exposure to TCDD significantly reduced serum estradiol in 2F males but not 2M males, and also significantly interfered with initial budding and subsequent growth of the prostate (particularly in the dorsal-lateral region) in 2F but not 2M males. In sharp contrast, the seminal vesicles were larger in control 2M males than in control 2F males, similar to prior findings in mice (Nonneman *et al.,* 1992), and TCDD only decreased the size of the seminal vesicles in 2M males.

MATERIALS AND METHODS

Animals. Time mated Holtzman rats were purchased from Harlan Sprague-Dawley Inc. (Madison, WI). Pregnant Holtzman rats were administered a single po dose of TCDD (1.0 μg/kg) dissolved in 95% corn oil and 5% acetone (2 ml/kg), or vehicle, on the morning of GD 15 (GD 0 = sperm positive).

On the morning of GD 20, fetuses were removed by caesarian section, and the intrauterine position of each animal was noted prior to collection of blood for steroid radioimmunoassays and removal of the urogenital complex for reconstruction analysis. Male fetuses residing *in utero* between 2 male fetuses (2M), between a male and a female fetus (1MF), and between 2 female fetuses (2F) were examined.

Fetuses from untreated dams were also collected on the morning of GD 18, 19, 20, and 21 in a separate experiment to examine the temporal pattern of prostate morphogenesis in this strain of rat. In the latter experiment, only 1MF male fetuses were examined to control for variability due to intrauterine position (n = 2 per time point).

Estradiol radioimmunoassay. Estradiol radioimmunoassay was performed as previously described (vom Saal *et al.*, 1990). Briefly, [^{125}I] estradiol and antisera were obtained from ICN Biomedicals (Costa Mesa, CA), and unlabeled estradiol was obtained from Steraloids (Wilton, NH). Sensitivity of the assay was 0.5 pg. Intra- and interassay coefficients of variation were 3 and 11%, respectively. We determined the percent cross-reactivity of the estradiol antiserum with estrone to be 0.6%. Cross-reactivity with other steroids was reported by ICN to be negligible.

Testosterone radioimmunoassay. Testosterone was assayed as described (vom Saal *et al.*, 1990). Briefly, first antibody (rabbit anti-testosterone), [^{125}I]testosterone, and second antibody (goat anti-rabbit) were obtained from ICN Biomedicals (Costa Mesa, CA). Intra- and interassay coefficients of variation were determined to be 3 and 12%, respectively. We determined the cross reactivity of the antisera with DHT and androstenedione to be 1.3% and 10%, respectively. Cross-reactivity with other steroids was reported by ICN to be negligible.

Reconstruction analysis. Rat fetuses were euthanized by decapitation and the entire urogenital complex, which includes the bladder, UGS, and associated accessory sex glands, was fixed overnight at 4°C in Bouin's solution. Fixed tissues were processed for histological examination and serial section reconstruction (Timms *et al.*, 1994). Epithelial outgrowths of the UGS called prostatic buds were categorized into ventral, lateral and dorsal and dorsocranial anatomical regions and analyzed by previously reported techniques (Roman *et al.*, 1998; Timms *et al.*, 1999; vom Saal *et al.*, 1997). Parameters measured for these regions included mean cross sectional area (CSA), total area of budding (TA), which is comparable to prostate volume, number of buds in each region, and length of budding (LB) along the UGS in a particular region of the prostate (Timms *et al.*, 1999). In addition to the prostate, the developing seminal vesicles associated with the proximal Wolffian ducts were also examined. For both the dorsocranial prostatic buds (also referred to as the coagulating glands) and the seminal vesicles, LB represents the mean distance in μm that the individual bud forming the organ extended from the urethra (dorsocranial buds) or Wolffian ducts (seminal vesicles); see Figures 1 and 2.

Morphometric Parameters

The regions of the developing urogenital complex (prostate and seminal vesicles) that we measured on GD 20 are depicted in Figure 2A, while the parameters measured for the dorsal, lateral, and ventral prostate (TA, mean CSA, and LB) are depicted in Figure 2B. These measures appear to be sensitive markers of hormonal effects on prostate budding morphogenesis (Timms *et al.*, 1999). The morphometric terms used in this study are based upon the following descriptions:

LB (in μm). LB was the length of UGS along which there were prostatic buds present. This was calculated from the number of sections multiplied by the thickness of sections (7 μm) of the UGS that contained buds associated with a specific region, since transverse sections through the UGS were examined. For the seminal vesicles and dorsocranial prostate (coagulating glands), the LB measure was the length of the developing structure, because the direction of growth of the individual structure forming these organs was perpendicular to the plane of section.

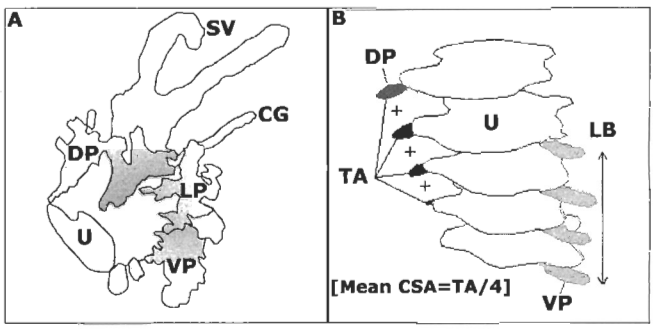

FIG. 2. (A) The anatomical regions of the developing prostate identified in this diagram were used to determine morphological parameters for this study. Abbreviations are the same as in Figure 1 legend. (B) For measurement of length of budding (LB) along the UGS, the number of sections associated with a structure (e.g., a ventral prostate bud—VP) is multiplied by the section thickness. For total area of budding (TA), a sum of the area of each individual bud profile (e.g., a dorsal bud) is performed. From the measured total area, the mean CSA is calculated by dividing TA by the number of sections associated with that particular structure (DP). See text for an additional explanation of morphological parameters.

CSA (in μm^2). The mean CSA was calculated by first summing the cross-sectional area measures for each bud identified in individual sections through a specific region, and then dividing by the number measured.

TA (in μm^2). TA was calculated as the sum of all of the individual cross-sectional areas for all of the buds in a specific region. This parameter serves as an estimate of volume.

Statistical analysis. Data were analyzed by ANOVA using SAS (GLM procedure). Because each litter contributed males from different IUPs, variance due to litter (maternal) effects was assessed by including litter as a main effect variable. The F value for IUP, treatment and the interaction was divided by the F value for the litter variable to generate a corrected F to determine significance in the overall ANOVA. Planned comparisons were conducted when the overall ANOVA was statistically significant using the LS means test in SAS, again after adjusting for variance due to litter effects. Planned comparisons consisted of comparing males within each treatment group (TCDD and control) for differences due to intrauterine position, and comparing treated versus untreated males from each intrauterine position. The confidence level for statistical significance was set at $p < 0.05$.

RESULTS

Time-Course Study of Prostate and Seminal Vesicle Morphogenesis

On GD 18 small ventral epithelial outgrowths in the caudal aspect of the UGS are the earliest indication of prostatic morphogenesis (Figs. 1 and 2). By this time in gestation, the seminal vesicles have already begun to form, as previously described (Schlegel *et al.*, 1967). Subsequently, continued growth on GD 19 occurs in the caudal UGS for both dorsal and ventral buds, and formation of additional buds in the lateral region also occurs toward the cranial aspect of the UGS. On

TABLE 1

Effect of *in Utero* TCDD Exposure on the Number of Prostatic Buds in the Dorsal, Lateral, and Ventral Regions of the Urogenital Complex of the Male Rat Fetus on GD 20

	Dorsal		Lateral		Ventral	
IUP	Control	TCDD†	Control	TCDD†	Control	TCDD
2F	32 ± 3	23 ± 3*	50 ± 4	34 ± 4**	16 ± 1	10 ± 1***
1MF	24 ± 3	22 ± 3	50 ± 4	42 ± 4	8 ± 1	8 ± 1
2M	38 ± 3	31 ± 3	47 ± 4	46 ± 4	17 ± 1	12 ± 1***
2F + 1MF + 2M	31 ± 2	25 ± 2*	49 ± 2	41 ± 2**	14 ± 1	10 ± 1***

Note. Individual buds were identified by region after section tracing and total numbers of buds were counted. 2F + 1MF + 2M – (morphological analysis that represents combined data from all 3 intrauterine positions). Statistical significance was determined by ANOVA (mean ± SEM, 8 or 9 litters, 5–6 males per litter).

*Significantly different from control, $p < 0.05$.

**Significantly different from control, $p < 0.01$.

***Significantly different from control, $p < 0.001$.

†Intrauterine position (IUP) comparisons: 2F and 2M males within a treatment group were significantly different ($p < 0.05$).

GD 20 most of the prostatic buds have formed, including those in the dorsocranial region that will form the coagulating glands. For this reason GD 20 was selected as a representative time point for examining the fetal prostate following TCDD treatment.

Number of Prostatic Buds

Exposure of fetal males to TCDD resulted in a significant 20% reduction in the total number of developing prostatic buds compared to untreated controls (TCDD = 76 ± 3 vs. control = 94 ± 4 [mean ± SEM]) on gestation day 20. A significant decrease in budding due to TCDD occurred in all regions (Table 1). The ventral region had the smallest population of developing buds, while the lateral region had the greatest number of developing buds.

Prostatic budding patterns were compared in control and TCDD-treated males, based upon both IUP and region (Table 1). A significant reduction in the number of prostatic buds in the TCDD-treated 2F males relative to control 2F males occurred in the dorsal, lateral, and ventral regions (29, 32, and 35% decrease, respectively). There was no significant reduction in TCDD-exposed 1MF males, and the only region showing a significant reduction in budding in 2M males was the ventral region. There were no differences between control 2F, 1MF, and 2M males in the number of prostatic buds in any region. However, as a result of the decrease in the number of buds that occurred for just the 2F males in dorsal and lateral regions, there was a significant difference between TCDD-exposed 2F and 2M males in the number of prostatic buds in these regions.

Morphometric Analyses

The data for the morphometric analyses for LB, mean CSA, and TA in the different regions of the prostate are presented in Table 2, and for the seminal vesicles and urethra in Table 3.

Dorsal Region of the Prostate

LB. Ignoring IUP, male fetuses exposed to TCDD showed a significant (17%) decrease in the length of the line of buds in the dorsal region relative to controls. The decrease observed in the TCDD-exposed animals was a consequence of a significant (27%) decrease in LB for only the 2F males. Control 2F males had a significantly shorter LB relative to control 2M males. TCDD-exposed 2F males also had a significantly shorter LB relative to TCDD-exposed 2M males.

CSA. Ignoring IUP, there was no significant difference in mean CSA due to TCDD treatment. For the control males mean CSA was 27% greater for 2F males relative to 2M males, replicating our prior finding with Sprague-Dawley rats (Timms *et al.,* 1999). In contrast, there was no significant difference between 2F and 2M males exposed to TCDD. Finally, TCDD had no significant effect on the mean CSA measure based on comparisons of TCDD-exposed and control males from each IUP.

TA. Ignoring IUP, there was no significant effect of TCDD on TA, but TCDD-treated 2F males showed a significant (33%) decrease in TA relative to control 2F males. Since control and TCDD-treated 2F males did not differ significantly in mean CSA, the effect of TCDD on TA was primarily due to a reduction in the length of the line of buds rather than a decrease in the cross-sectional area of the buds. In contrast to 2F males, the TCDD-exposed 1MF and 2M males did not differ significantly from control males from the same IUP on the TA measure (see also Figs. 3 and 4).

Lateral Region of the Prostate

LB. Ignoring IUP, male fetuses exposed to TCDD tended to show a decrease (by 16%) in the length of the line of buds in the lateral region relative to controls ($p = 0.09$). For controls, LB was somewhat longer for 2M than 2F males, but the

TABLE 2

Effect of *in Utero* TCDD Exposure on the Morphometric Parameters for the Anatomical Regions of the Prostate

	LB (μm)		Mean CSA (μm^2)		TA (μm^2)	
IUP	Control†	TCDD‡	Control‡	TCDD	Control	TCDD‡
Dorsal region of the prostate						
2F	861 ± 94.5	628 ± 86**	1150 ± 72	1054 ± 66	141,712 ± 9551	94,675 ± 8664**
1MF	787 ± 94.5	798 ± 88	1031 ± 72	1201 ± 67	113,633 ± 9,551	134,067 ± 8842
2M	1127 ± 90	881 ± 101	905 ± 69	1047 ± 77	139,913 ± 9,132	131,242 ± 10,210
2F + 1MF + 2M	925 ± 54	769 ± 53*	1029 ± 41	1101 ± 41	131,753 ± 5435	119,994 ± 5349
Lateral region of the prostate						
2F	1140 ± 166	743 ± 151*	1003 ± 69	973 ± 63	163,466 ± 28,950	105,013 ± 26,261*
1MF	1420 ± 166	1393 ± 154	1042 ± 69	1083 ± 64	213,956 ± 28,950	219,884 ± 26,803
2M	1369 ± 159	1161 ± 178	881 ± 66	889 ± 74	167,826 ± 27,682	145,911 ± 30,949
2F + 1MF + 2M	1309 ± 95	1099 ± 93	975 ± 39	982 ± 39	181,750 ± 16,474	156,936 ± 16,214
Ventral region of the prostate						
2F	564 ± 63	492 ± 57	2283 ± 221	2476 ± 201	184,440 ± 26,885	169,289 ± 24,388
1MF	406 ± 63	552 ± 58	2492 ± 221	3042 ± 205	147,955 ± 26,885	234,904 ± 24,891
2M	680 ± 60	510 ± 67	2567 ± 211	2738 ± 236	251,859 ± 25,707	203,348 ± 28,742
2F + 1MF + 2M	550 ± 35.7	518 ± 35	2447 ± 126	2752 ± 124	194,751 ± 15,299	202,514 ± 15,057
Dorsocranial region of the prostate (coagulating glands)						
2F	270 ± 28	209 ± 26*	1516 ± 84	1529 ± 76	58,415 ± 6,847	44,647 ± 6211*
1MF	291 ± 28	294 ± 26	1449 ± 84	1807 ± 78	60,066 ± 6,847	75,816 ± 6339
2M	315 ± 27	292 ± 30	1493 ± 80	1733 ± 90	66,567 ± 6,547	73,442 ± 7320
2F + 1MF + 2M	292 ± 16	265 ± 16	1486 ± 48	1690 ± 47**	61,683 ± 3,896	64,635 ± 3834

Note. IUP, intrauterine position; LB length of budding along the UGS; TA, total area of budding; mean CSA, mean cross sectional area in the dorsal, lateral, and ventral regions of the prostate as well as for the single pair of dorsocranial prostatic buds (coagulating glands). For the dorsocranial buds LB represents the mean distance in μm that the paired buds extend from the urethra. In contrast, for the dorsal, lateral, and ventral regions of the prostate, LB represents the length of the urogenital sinus that is associated with the buds in each anatomical region. Values are mean ± SEM; n = 5–6 for IUP; 80–90 sections were used for morphological parameters.

*Significantly different from control, $p < 0.05$.

**Significantly different from control, $p < 0.01$.

***Significantly different from control, $p < 0.001$.

†IUP comparisons: 2F and 2M males within a treatment group were significantly different ($p < 0.05$).

‡IUP comparisons: 2F and 2M males within a treatment group were significantly different ($p < 0.06$).

difference was not statistically significant. For TCDD-exposed males, the length of the line of buds was significantly greater for 2M than for 2F males. TCDD resulted in a significant decrease in LB (by 35%) for only the 2F males.

CSA. TCDD had no significant effect on the mean CSA measurement; either based on comparisons of treated and control males from each IUP or ignoring IUP. The mean CSA of budding was slightly greater for control 2F males relative to control 2M males, but the difference was not statistically significant.

TA. Ignoring IUP, there was no significant effect of IUP or TCDD on the TA measure for the lateral prostatic region of budding. However, similar to the dorsal prostate, TCDD significantly reduced TA in 2F males but not 1MF or 2M males (see also Figs. 3 and 4).

Dorsocranial Region of the Prostate (Coagulating Glands)

Length of the paired glands. The coagulating glands in fetal rats develop as a single pair of buds located in the most cranial region of the dorsal prostatic urethra. Note that for these buds, the length measure represents the mean distance in μm that the paired buds, which form the adult coagulating glands, extend from the urethra.

Ignoring IUP, male fetuses exposed to TCDD did not show a statistically significant, decrease in the length of these bilateral dorsocranial buds relative to controls. For the comparison of males within each treatment group from different IUPs, the length of the dorsocranial pair of buds did not differ significantly as a function of IUP for control males. However, for TCDD-treated males the length of the pair of the buds was significantly greater for 2M than for 2F males. The basis for this finding is that for the TCDD-exposed 2F males, there was a significant decrease in bud length relative to control 2F males, while there was no decrease in bud length due to TCDD for 2M or 1MF males relative to controls from the same IUP. TCDD thus only decreased the length of the dorsocranial buds in 2F males.

TABLE 3

Effect of *in Utero* TCDD Exposure on the Morphometric Parameters for the Seminal Vesicles and Prostatic Urethra

	LB (μm)		Mean CSA (μm^2)		TA (μm^2)	
IUP	Control‡	TCDD	Control	TCDD	Control‡	TCDD
Seminal vesicles						
2F	477 ± 45	473 ± 41	4891 ± 462	4475 ± 419	332,819 ± 34,860	300,381 ± 31,622
1MF	473 ± 45	540 ± 41	4968 ± 462	4532 ± 427	337,013 ± 34,860	340,067 ± 32,274
2M	657 ± 43	493 ± 48*	4768 ± 441	4612 ± 493	435,706 ± 33,333	325,779 ± 37,267*
2F + 1MF + 2M	536 ± 25	502 ± 25	4876 ± 263	4540 ± 259	368,513 ± 19,837	322,076 ± 19,523
Urethra						
2F	256 ± 7	244 ± 14	74,327 ± 2661	98,645 ± 15,983		
1MF	305 ± 24	336 ± 21	71,152 ± 8880	72,881 ± 9166		
2M	344 ± 27	272 ± 20*	67,840 ± 2685	83,758 ± 7597		
2F + 1MF + 2M	302 ± 12	284 ± 11	71,106 ± 3063	85,094 ± 6750		

Note. IUP, intrauterine poisition; LB (seminal vesicles), mean distance in μm that the developing glands extend from the urethra; LB (urethra), the length associated with prostatic budding from the cranial to caudal portion of the UGS; mean CSA, mean cross sectional area; TA, total area of budding. Values are mean ± SEM; n = 5–6 for IUP; 80–90 sections were used for morphological parameters.

*Significantly different from control, $p < 0.05$.

**Significantly different from control, $p < 0.01$.

***Significantly different from control, $p < 0.001$.

†IUP comparisons: 2F and 2M males within a treatment group were significantly different ($p < 0.05$).

‡IUP comparisons: 2F and 2M males within a treatment group were significantly different ($p < 0.06$).

CSA. Ignoring IUP, the mean CSA of the bilateral dorsocranial buds was greater for TCDD-treated relative to control males. There were no significant differences due to IUP for males within each treatment group in the mean CSA measure.

TA. Ignoring IUP, there was no significant difference in the TA measure between TCDD and control males. In addition, control 2F, 1MF, and 2M males did not differ in the total area of budding. However, as described above, the TCDD-treated 2F males had shorter buds relative to control 2F males, while TCDD treatment did not affect bud length in control 2M males. As a result, there was a significant difference in TA between control and TCDD-treated 2F males, but not 1MF or 2M males. In addition, there was a significant difference between 2F and 2M TCDD-treated males, with the TA measure being significantly smaller for TCDD-treated 2F males relative to 2M males.

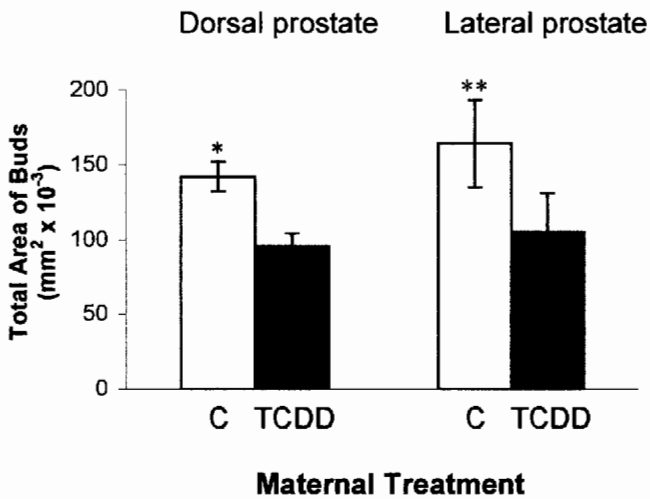

FIG. 3. Total area of glandular buds (TA) in the dorsal and lateral regions of the prostate of the 2F male rat fetus on GD 20. The 2F males, but not the 2M males (data not shown), showed a significant decrease in TA in these regions of the developing prostate. Mean ± SEM; *$p < 0.005$; **$p < 0.05$; n = 5–6 for IUP; 80–90 sections were used for morphological parameters.

Ventral Region

There were no significant differences in the LB, mean CSA, or TA measures based on comparisons of males from different IUPs or due to TCDD treatment in the ventral prostatic region, even though there were significantly fewer buds in TCDD-treated males relative to control males (Table 1).

Seminal Vesicles

Length of the paired glands. As was described for the length measure in the developing coagulating glands, the seminal vesicle length measure represents the length of the paired glands as they extend from the ejaculatory (Wolffian) ducts. Ignoring IUP, there was no effect of TCDD on the length, mean CSA, or TA measures for the seminal vesicles. However, for control males, the length of the seminal vesicle was significantly greater in 2M males (by 35%) relative to 2F males. TCDD treatment significantly reduced seminal vesicle length (LB) in 2M males, but not in 1MF or 2F males. As a result, for TCDD-treated males, there was no difference between 2F, 1MF, and 2M males for the LB measure.

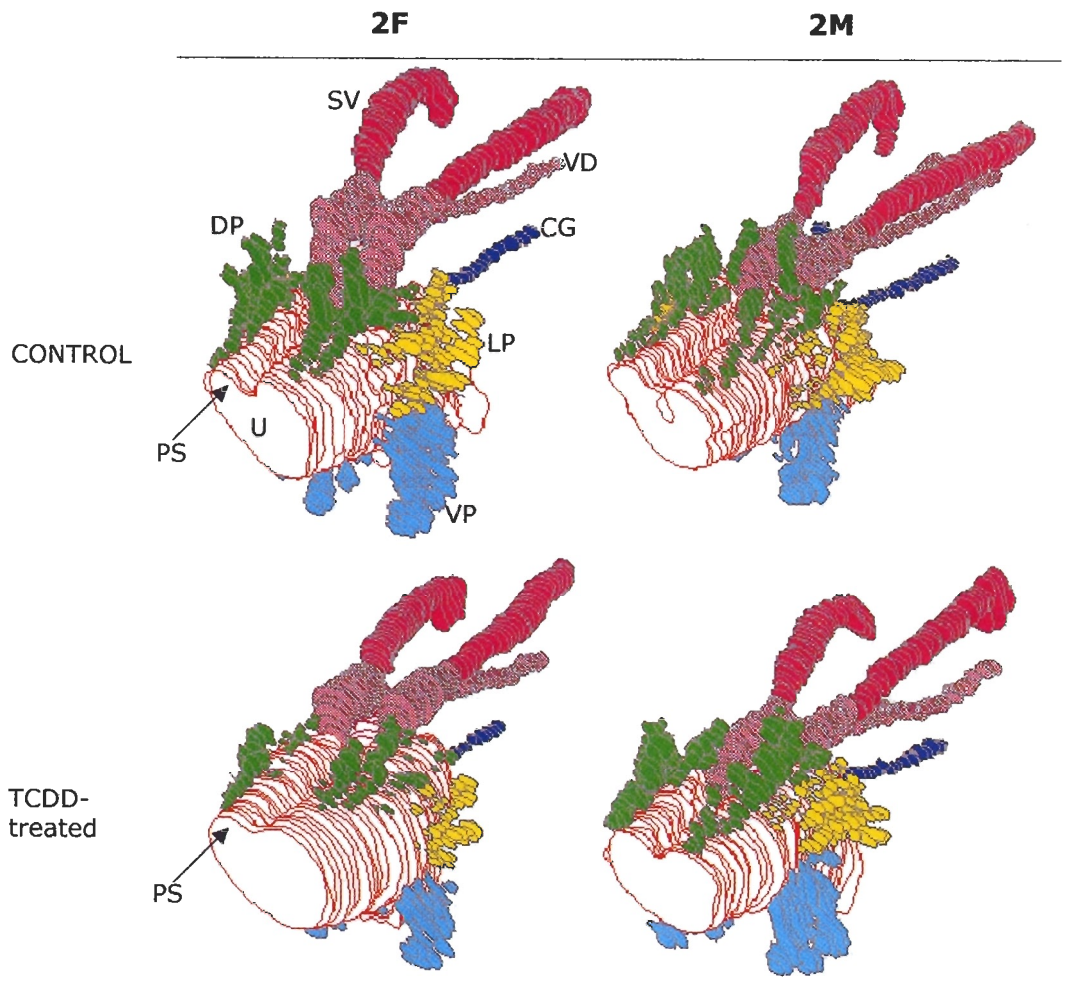

FIG. 4. Serial section reconstructions of the fetal rat UGS on GD 20. Abbreviations are the same as in Figure 1 legend. A comparison of the prostate budding pattern between the 2F and 2M control males illustrates the effect of intrauterine position on increased size of the prostate (see Table 2, Dorsal Region of the Prostate, for comparison showing mean CSA larger for 2F than 2M males in the dorsal region of the prostate). By comparison, TCDD-treated 2F males have significantly reduced prostate growth, particularly in the dorsal and lateral regions, relative to 2F control males. In sharp contrast, TCDD-treated 2M males do not exhibit significantly different growth patterns from 2M controls. Of interest is the effect of TCDD-treatment on the shape of the urethra, notably in the 2F males. In these animals the prostatic sulcus (PS) is less defined that that of the control males.

CSA. There was no significant difference due to treatment or IUP in the mean CSA of the seminal vesicles.

TA. Ignoring IUP there was no significant effect on seminal vesicle TA due to TCDD treatment. However, the TA for control 2M males was significantly greater than for control 2F males. Maternal TCDD treatment significantly reduced TA in 2M males, but not in 1MF or 2F males. The significant decrease in TA in 2M males exposed to TCDD was due to a significant decrease in the length of the gland.

Urethra

The region of the urethra in which there were prostatic buds (prostatic urethra) was significantly longer in control 2M males (by 35%) relative to control 2F males ($p < 0.01$). TCDD resulted in the prostatic urethra of 2M males being significantly shorter (by 17%) relative to control 2M males ($p < 0.05$). Ignoring IUP, the mean CSA of the prostatic urethra tended to be larger in TCDD-treated males than in control males ($p = 0.09$). Furthermore, TCDD tended to increase mean CSA in 2F males ($p = 0.09$), but not in 1MF or 2M males. There was also a noticeable change in the shape of the urethral contour in TCDD-exposed fetuses. The prostatic sulci (Fig. 4) in TCDD-exposed males were less pronounced compared to controls.

Prostatic utricle. The prostatic utricle is the remnant of the Müllerian ducts as they enter the UGS, and this remnant persists in the dorsal region of the prostate. There was a significant decrease in the length of the utricle (LB) in TCDD-treated males (72 ± 5 μm) relative to control (92 ± 6 μm; $p < 0.05$). There were no differences observed relating to IUP for the utricle for control or TCDD-treated males (data not shown).

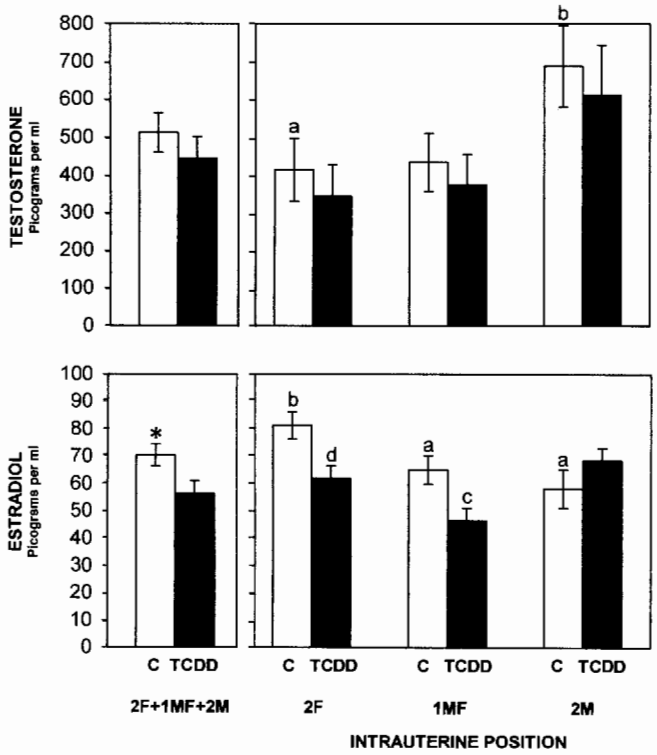

FIG. 5. Serum testosterone and estradiol in all control (C) and TCDD-treated (TCDD) male fetuses on GD 20 (2F + 1MF + 2M), as well as hormone levels for 2F, 1MF, and 2M males. Control 2M males had higher levels of testosterone than did 2F males, but there was no significant effect of TCDD on serum testosterone. Serum estradiol was significantly higher in 2F males than in 2M males, and while TCDD did not alter serum estradiol in 2M males, TCDD caused a significant reduction in serum estradiol in 2F and 1MF males. (Mean ± SEM; $*p = 0.06$; a vs. b, $p < 0.05$; b vs. d, $p < 0.01$; a vs. c, $p < 0.05$; $n = 5$–6).

Serum Estradiol and Testosterone

In control males serum estradiol was significantly greater in 2F males than in either 1MF males or 2M males (Fig. 5). For TCDD-treated males, 2F and 2M males did not differ significantly in their serum estradiol levels. Ignoring IUP, serum estradiol in TCDD-exposed males tended to be lower than in control males ($p = 0.06$). Relative to control 2F males, TCDD-exposed 2F males had significantly lower serum estradiol. Similarly, relative to control 1MF males, TCDD-exposed 1MF males also had significantly lower serum estradiol. However, TCDD did not have a significant effect on serum estradiol in 2M males.

In control males serum testosterone was significantly greater in 2M males than in either 2F males or 1MF males. For TCDD-treated males, serum testosterone tended ($p = 0.09$) to be higher in 2M than in 2F males. Ignoring IUP, TCDD did not significantly alter serum testosterone levels. Serum testosterone did not differ significantly based on comparisons of control males and TCDD-treated males from each IUP (Fig. 5).

DISCUSSION

There are two major findings from this study. First, ignoring IUP, fetal exposure to TCDD caused a 20% reduction in the total number of prostatic buds that develop from the fetal UGS. However, this reduction was primarily due to a selective reduction (31%) in prostatic buds by TCDD in 2F males, who are exposed naturally to the highest levels of circulating estradiol during fetal development. In contrast, 2M male fetuses, with the lowest serum estradiol levels, showed only a small (not statistically significant) 10% decrease in the number of prostatic buds. In addition, 2F males (but not 2M males) that were exposed to TCDD showed a significant decrease in the size of the prostatic buds relative to controls from the same IUP. However, this inhibitory effect of TCDD on the growth of prostatic glandular buds in 2F males was restricted to the dorsocranial, dorsal, and lateral regions. No significant effect on growth of glandular buds in the ventral region was observed for males from any intrauterine position. The interesting aspect of this finding is that the rodent dorsocranial, dorsal, and lateral regions of prostatic buds are comparable to buds that form similar regions in the developing human prostate. Unlike the rodent prostate, there is no portion of the adult prostate in humans that derives from the ventral region of the UGS (Timms, 1997).

The loss of prostatic buds due to TCDD exposure was primarily in the caudal region of the prostate. The findings concerning the initial pattern of bud development beginning on GD 18 (Fig. 1) suggest that the inhibitory effect of TCDD on the overall growth of prostatic buds from the UGS does not involve retardation in the rate of budding. This hypothesis is based on the fact that the first buds to form between GD 18 and 19 are in the caudal region of the prostate, followed between GD 19 and 20 by formation of buds in the cranial region. A retardation in the rate of growth by TCDD based on examination on GD 20 would thus be expected to result in a delay in the development of buds in the cranial region of the prostate, not in the caudal region. Since the prostates in TCDD-exposed males did not resemble control prostates collected on GD 19, we conclude that TCDD did not retard bud development. Instead, these findings show that the effect of TCDD on the prostate depends on the background level of estradiol in male fetuses, and that TCDD shows regional specificity in disrupting prostatic bud development and growth within the differentiating UGS.

The second major finding is that the effect of TCDD on the prostate in 2F males was associated with a significant decrease in serum estradiol in these 2F males, while their 2M male siblings were unaffected by TCDD both in terms of serum estradiol levels and dorsocranial, dorsal, and lateral prostate glandular bud number and size. These findings lead to a number of hypotheses. First, the inhibitory effect of TCDD on serum estradiol is modulated by the background level of estradiol present at the time of exposure to TCDD. In addition,

the selective effect of TCDD only on the prostate of male fetuses that would otherwise have had high levels of serum estradiol (and an enlarged prostate) may have been due, at least in part, to the decrease in estradiol in addition to a likely direct effect of TCDD on the prostate. However, in the C57B1/6 mouse fetus, *in utero* exposure to TCDD has been shown to impair prostatic bud formation by an AhR-dependent process (Lin *et al.,* 2000). Thus, at least some of the effects reported here are likely also due to activation of the aryl hydrocarbon receptor (AhR) in mesenchymal and/or epithelial cells of the UGS. Both AhR and the AhR nuclear translocator protein (ARNT) are expressed in the fetal rat UGS during the time that TCDD would have been acting as a result of maternal treatment on GD 15 (Sommer *et al.,* 1999). In the 2M fetuses, testosterone levels were high in both the TCDD and control animals, compared to 2F fetuses. There is a possibility that higher circulating levels of testosterone may provide protection for the developing prostate from the growth inhibitory effects of TCDD. If TCDD interacts with AhR and produces factors that inhibit prostate development or antagonize androgen-driven processes, then a higher level of testosterone may help to modulate this effect.

The developing prostate is primarily responsive to androgen during development (Cooke *et al.,* 1991; Prins and Birch, 1995). Thus, prior studies of the inhibitory effect of TCDD on prostate development in rats had focused on the possible effects of TCDD on serum testosterone (Roman and Peterson, 1998; Roman *et al.,* 1995; Theobald *et al.,* 2000a,b). We confirmed here that circulating testosterone is unaffected by TCDD in male rat fetuses. However, there is increasing evidence that estrogen plays an important role in both normal development and subsequent abnormal growth of the prostate gland (Ho *et al.,* 1992; Prins, 1997; vom Saal and Timms, 1999; vom Saal *et al.,* 1997). We previously reported that the pattern of prostatic budding from the UGS can be altered by experimentally manipulating the levels of circulating estradiol during critical periods in fetal development in mice. Specifically, increasing serum estradiol in male mouse fetuses by 50% resulted in an increase in the number and size of prostate glands, and a permanent increase in prostatic androgen receptors, relative to untreated males (vom Saal *et al.,* 1997). In addition, the natural phenomenon of IUP exposes developing fetuses to variable levels of circulating estradiol and testosterone. The consequences of IUP on rat prostate development, namely an increase in the size of the dorsolateral region of the developing prostate in 2F males, adds further credence to the hypothesis that estradiol enhances androgen regulation of prostatic growth in a region-specific manner (Timms *et al.,* 1999). In mice, 2F males also have permanently enlarged prostates relative to 2M males (Nonneman *et al.,* 1992).

For control male fetuses, we replicated prior findings in mice (vom Saal, 1989) and Mongolian gerbils (Clark *et al.,* 1991) that serum estradiol is higher in 2F than in 2M male fetuses, while serum testosterone is higher in 2M than 2F male fetuses. This difference in serum hormone levels due to being positioned between either male or female fetuses has been shown to be mediated by transport through the amniotic fluid and across the amniotic and chorionic (fetal) membranes surrounding each fetus; the fetal membranes are pressed against each other toward the end of gestation (Even *et al.,* 1992). In contrast to primates, where the placenta contains aromatase and secretes estrogen (Solomon, 1994), the rat placenta secretes androgen (androstenedione and testosterone) but not estrogen (Jackson and Albrecht, 1985; Sridaran and Gibori, 1987; Warshaw *et al.,* 1986). It is interesting that TCDD did not significantly decrease serum testosterone, but did significantly lower serum estradiol in male fetuses. Why serum estradiol was selectively not reduced in 2M males, while there was a significant reduction in estradiol in 1MF and 2F males whose mothers were treated with TCDD, remains to be determined.

In previous comparisons of 2F and 2M male mice, while 2F males with elevated serum estradiol during fetal life were found to have a permanently enlarged prostate, these same males had seminal vesicles that were permanently reduced in size (Nonneman *et al.,* 1992). Subsequent studies have revealed that the small seminal vesicles in 2F males were due to lower 5α-reductase activity relative to 2M males (Ganjam, Welshons, and vom Saal, unpublished observation), while no difference in seminal vesicle androgen receptors was observed (Nonneman *et al.,* 1992). In addition, administration of estrogenic chemicals, such as bisphenol A, to pregnant mice resulted in a decrease in seminal vesicle and epididymis size in male offspring and an increase in prostate size relative to controls (Gupta, 2000a; vom Saal *et al.,* 1998). These findings show that during fetal life, gonadal steroids have opposite effects on the development of organs that differentiate from the Wolffian ducts and the UGS. Our finding here that 2M male Holtzman rats had larger seminal vesicles than 2F males is consistent with these prior findings in mice. Interestingly, this is not consistent with our prior comparison of 2F and 2M Sprague-Dawley rats (Timms *et al.,* 1999). In addition, the fact that TCDD disrupted development of the seminal vesicle in 2M males but not 2F males suggests that there is a marked difference in the interaction between TCDD and gonadal steroids in influencing seminal vesicle development in contrast to prostate development.

The mean cross sectional area of the lumen of the urethra associated with prostatic buds (prostatic urethra) was decreased in a prior study in which serum estradiol was experimentally increased in male mouse fetuses (vom Saal *et al.,* 1997). In this study, ignoring IUP, TCDD tended to increase the mean cross sectional area of the prostatic urethra, and this was associated with a decrease in serum estradiol in these males. Development of the urethra is thus sensitive to changes in estrogen. In addition, TCDD treatment resulted in a change in the shape of the dorsal portion of the UGS, such that there was a noticeable decrease in the depth of the prostatic sulcus (Fig. 4). This

portion of the fetal UGS is the region from which the buds that form the dorsal prostate differentiate. This change in shape could thus be involved in the loss of dorsal prostatic buds from the UGS. In addition, changes in the morphology of the urethra could result in subsequent abnormalities in urethral function, which remain to be examined.

Taken together, our findings demonstrate that *in utero* exposure to TCDD disrupts the development of the prostate, but this disruption depends on an interaction with background levels of estradiol. There are numerous factors that influence levels of estradiol in human pregnancy, such as race, birth order, fetal body weight, and singleton versus twin pregnancy (Batra *et al.*, 1978; Bernstein *et al.*, 1986; Gerhard *et al.*, 1987; Henderson *et al.*, 1988). Our findings suggest that variation in estradiol in human fetuses might also be a factor that influences the response to TCDD in humans. The interaction between TCDD and estradiol leads to the hypothesis that the effects of TCDD might also be altered by simultaneous exposure to estrogenic chemicals, which are found in plastics, pesticides, and other products. The potential for interactive effects of these chemicals on prostate growth and development requires that studies of mixtures of these chemicals be conducted.

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