Prenatal TCDD Exposure Delays Differentiation and Alters Cell Proliferation and Apoptosis in the Uterus of the Sprague-Dawley Rat

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Abstract: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an endocrine-disrupting chemical that alters cellular organization at both macroscopic and molecular levels. Our goal was to determine the effects that prenatal TCDD exposure has on uterine morphology, cell proliferation, apoptosis, and protein expression. Pregnant Sprague-Dawley rats were treated with 3 μg TCDD/kg body weight by gavage on gestational day 15. At 50 days postpartum, female offspring exposed *in utero* to TCDD displayed uteri that were atrophic in appearance, but with a 2-fold significant increase in luminal epithelial cell proliferation and a significant decrease in apoptosis (10- and 4-fold in glandular and luminal epithelium, respectively), compared to the controls. Epidermal growth factor receptor (EGFR) was significantly increased and superoxide dismutase 1 (SOD1) was significantly decreased in uteri of rats exposed prenatally to TCDD. We conclude that TCDD can inhibit maturation and modulate uterine proteins that are known to play a role in uterine growth as well as alter epithelial cell proliferation and apoptosis in a manner that may enhance disease, including carcinogenesis.

Key Words: TCDD, uterus, proliferation, apoptosis.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an extremely toxic environmental contaminant that is formed during industrial incineration of wastes containing polychlorinated benzenes and chlorophenoxy ethers [1]. It is often referred to as the prototype of the dioxin family and as the most toxic, man-made chemical [2]. Over the past two decades, much research has been focused on TCDD due to its potential for endocrine disruption and its designation as a known human carcinogen, as proclaimed by the International Agency for Research on Cancer (IARC) in 1997. In a recent review, it was reported that TCDD and other dioxins cause effects at all levels of biological organization, affecting metabolism, macroscopic organ and tissue function, cellular communication mechanisms, and enzyme function [3].

In April of 2006, the National Toxicology Program published a technical report on the long term effects of TCDD [4]. Animals that were exposed to TCDD orally for two years displayed a number of diseases, including cancers of the liver, lung, oral cavity and uterus. This report followed the work of Rier *et al.* who demonstrated an increased incidence of endometriosis in rhesus monkeys following chronic dietary exposure to TCDD [5].

The female reproductive organs appear to be especially sensitive to exposures of TCDD. It has been reported that a number of important changes were present in the female reproductive tract after prenatal exposure to TCDD [6]. Rats exposed to TCDD on gestational day 15 developed ovarian neoplasms, Sertoli cell tumors in the female reproductive tract and an increased incidence and severity of ovarian interstitial hyperplasia. Additionally, growth was reduced at prenatal doses of 0.8 and 1.0 μg TCDD/kg body weight (BW) administered by gavage. There have been reported gross malformations of the external genitalia of female rat offspring exposed to TCDD *in utero* [7]. Female rats exposed perinatally to TCDD weighed significantly less than the control rats and displayed delayed vaginal opening and persistent vaginal threads [8]. Our lab has shown that prenatal TCDD treatment can have carcinogenic consequences in the mammary gland, another hormone-responsive organ. TCDD exposure led to a two-fold increase in mammary tumors per rat using a 7,12-dimethylbenz(a)anthracene (DMBA)-induced model [9,10]. In that study, an increased number of terminal end buds, a proliferative, immature structure that is susceptible to carcinogenesis, were observed at the time of DMBA exposure in TCDD-exposed animals, suggesting that TCDD delayed mammary gland maturation. Other investigators have noted that mammary glands taken from 4-day-old offspring exposed perinatally to TCDD had reduced primary branches, decreased epithelial elongation, and significantly fewer alveolar buds and lateral branches [8]. Though control animals developed well-differentiated terminal structures by postnatal day 68, TCDD-exposed animals retained undifferentiated terminal structures.

One pathway containing tyrosine kinases that has been implicated in the regulation of uterine proliferation and apoptosis is the epidermal growth factor (EGF) pathway [11]. EGF and its receptors, the ErbB family of tyrosine kinases that includes epidermal growth factor receptor (EGFR), form a network that is known to play a role in cell proliferation in

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many tissue types. EGF pathway activation has been associated with cell proliferation or a block of apoptosis in several tissues [reviewed in 12]. Interestingly, mice deficient in EGF are not susceptible to certain TCDD toxicities, highlighting the importance of the EGF pathway after TCDD exposure [13]. Several reports have observed an increase of transforming growth factor-alpha (TGF-α), a known ligand for the EGFR, following TCDD exposure [14]. Our lab has also shown an association between EGFR levels and tissues (mammary and uterus) that are undergoing cell proliferation [15,16].

The many toxic effects of TCDD have been observed in a host of different species and in multiple organs. There have been several pathways and signaling molecules implicated in these toxic effects. A recent review article points to the importance of cellular stress responses in the toxic actions of TCDD [12]. After TCDD exposure, an increase in superoxide anion production has been observed. Prostaglandin synthesis and the production of inflammatory cytokines are also observed in many organs. These observations necessitate studying the role of enzymes that regulate the levels of reactive oxygen species, such as superoxide dismutase (SOD).

TCDD is well established as an endocrine disruptor and a carcinogen for several organs, including the uterus. A report by Li *et al.* demonstrates that TCDD may accumulate in the uterus and contribute to disease [17]. The mechanisms that can lead to uterine disease following TCDD exposure have not been elucidated at this time. The goal of this study was to determine whether TCDD administered on day 15 of gestation, a critical period of fetal organ development, could alter uterine architecture, differentiation, and proliferation/apoptosis at both pre- and post-pubertal time points. We also looked at the modulation of proteins that are known to play a role in cell proliferation in the uterus. The EGF pathway is known to play a role in uterine proliferation and growth, as well as disease susceptibility. We also measured SOD1, the primary scavenger of superoxide anion radicals, which are often observed after TCDD exposure. We hypothesize that prenatal exposure to TCDD can alter uterine differentiation and create an environment that leaves the uterus more susceptible to disease, including cancer.

MATERIALS AND METHODS

Animals

Animal studies were performed according to the guidelines and protocols approved by the UAB Institutional Animal Care and Use Committee. We purchased female Sprague-Dawley CD rats from Charles River Breeding Laboratories (Raleigh, NC). All animals were fed AIN-93G base diet (Harlan Teklad, Madison, WI). AIN-93G is a purified diet containing no detectable estrogens. Sixty female rats were bred and the date of conception for each female (when sperm is present in the vagina) was determined by daily vaginal smears according to a previously outlined protocol [18]. Animals were maintained with food and water available *ad libitum*. Polypropylene cages and water bottles were used for housing the rats and for the drinking supply. Animals were maintained on a light:dark cycle (12:12) with lights on at 0800 hr and off at 2000 hr. The animal room temperature was maintained at 22 °C.

At days 21 and 50 postpartum, female offspring were weighed and subsequently anesthetized using ketamine and xylazine. At 50 days postpartum, all rats were sacrificed in the estrous phase of the cycle. Following sedation of the rats, live collections of the uteri were performed in order to minimize protein degradation. Tissues collected were weighed and paraffin blocked or frozen in liquid nitrogen until tissue processing. Animals were subsequently decapitated and trunk blood was immediately collected. The blood was centrifuged at 2300 revolutions per minute for 15 min, and serum was collected and frozen at –80 °C.

TCDD Exposure

Pregnant females were treated with TCDD (Cambridge Isotope Laboratories Inc., Andover, MA) by gavage at a concentration of 3 μg/kg BW. The TCDD treatment was administered on day 15 post-conception (recognition of sperm in vagina designated as day 1). Controls received an equivalent volume of sesame oil on the same schedule. At birth, all pups were cross-fostered to lactating rats not exposed to TCDD to prevent postpartum TCDD exposure.

Uterine Morphology and Epithelial Cell Height

5 μm sections from paraffin-blocked uteri from 50-day-old rats were cut onto glass slides and subsequently stained using a standard hematoxylin and eosin (H&E) staining protocol. These H&E stained uteri were evaluated for histological and morphological changes by a board-certified pathologist.

Uterine luminal epithelial cell height was also measured in the uteri of rats at 50 days postpartum. H&E stained sections were evaluated *via* a Nikon light microscope (with a 40x objective lens magnification) and Nikon digital camera. There were seven uteri evaluated per treatment group. From each uterus, five separate areas were imaged with four cell height measurements made per area. Images were analyzed for height using Image J software (National Institutes of Health, Bethesda, MD).

Cell Proliferation Analysis

Cell proliferation was evaluated using immunohistochemical (IHC) analysis of Ki67, a protein expressed in all phases of active cell cycle. Five-μm-thick tissue sections were cut from paraffin-embedded tissue blocks and mounted on glass slides. Paraffin-embedded uteri were de-paraffinized in xylene and rehydrated in a series of graded alcohols (100, 95, 70, and 50%). Samples were then washed in dH_2O followed by phosphate-buffered saline (PBS). Antigen retrieval was performed using the Vector Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). Specimens were boiled in the Antigen Unmasking Solution for 20 min and then allowed to cool to room temperature. Endogenous peroxidase activity was blocked by incubating specimens in 3% hydrogen peroxide at room temperature for 10 min. Blocking was done using ready-to-use 2.5% Normal Horse Serum from the ImmPRESS™ Reagent Kit (Anti-Mouse Ig) (Vector). Next, a monoclonal mouse anti-rat Ki67 antigen antibody (DakoCytomation, Carpinteria, CA) was applied to the specimens for 30 min followed by three washes in PBS. A ready to use ImmPRESS™ Reagent secondary antibody

(Vector) was applied to the samples for 30 min followed by washes in PBS. A Peroxidase Substrate Kit, 3,3' diaminobenzidine (DAB) (Vector), was applied to samples for 10 min to develop color, followed by a wash in tap water. To counterstain, hematoxylin QS (Vector) was applied to the specimens. Clearing was performed by immersing the specimens in a series of graded alcohols and then xylenes. Specimens were mounted and coverslips were applied using Vector Mounting Media. Visualization was performed using a Nikon light microscope (with a 40x objective lens magnification) and Nikon digital camera, and images were analyzed using Image J software. The glandular and luminal epithelial cells stained for Ki67 were counted as well as the total number of epithelial cells per uterine section (at least 1000 cells). Sections from six animals in both the control and treatment groups were analyzed. A proportion score (proliferative index) was given by the number of stained cells / total number of cells counted x 100.

Apoptosis Analysis

The ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) was used to measure apoptosis following the manufacturer's instructions. Five-μm-thick tissue sections were cut from paraffin-embedded tissue blocks and mounted on glass slides. Paraffin-embedded tissue sections were de-paraffinized and rehydrated in graded alcohols (100, 95, and 70%). Tissues were treated with freshly diluted Proteinase K (20 μg/mL) (Qiagen, Valencia, CA) for 15 min at room temperature and then washed in dH_2O. Endogenous peroxidases were inactivated with 3% hydrogen peroxide for 5 min and then washed in PBS. Equilibration buffer was added to the sample for 20 min, followed by a 1-hr application of Terminal Deoxynucleotidyl Transferase enzyme in a humidified chamber at 37°C. Next, stop/wash buffer was added, followed by 30 min incubation with an anti-digoxignenin conjugate at room temperature. Tissues were washed four times in PBS. To develop color, specimens were covered with DAB substrate diluted by DAB dilution buffer for 10 min at room temperature. Specimens were washed and then counterstained with 0.3% methyl green for 10 min. Next, sections were washed in three changes of dH_2O followed by three washes in 100% n-butanol. The specimens were cleared using graded alcohols and xylene and then mounted using Permount mounting solution and coverslips. The apoptotic index was defined as the number of epithelial cells stained positive for apoptosis (as observed by a brown staining color) divided by the total number of epithelial cells counted x 100. Separate indices were determined for glandular and luminal epithelia. Visualization was performed using a Nikon light microscope (with a 40x objective lens magnification) and Nikon digital camera, and images were analyzed using Image J software.

Western Blot Analysis

The following biomarkers were measured using western immunoblot analysis: estrogen receptor-alpha (ER-α) (C-311/sc-787) (Santa Cruz Biotechnologies, Santa Cruz, CA); estrogen receptor-beta (ER-β) (H-150/sc-8974) (Santa Cruz); progesterone receptor (PR) (C-19/ sc-538) (Santa Cruz); EGFR (1005/sc-03) (Santa Cruz); SOD1 (C-17/sc-8637) (Santa Cruz).

Uteri of animals were processed and western blot analysis was performed using a previously outlined protocol [19]. Samples were homogenized using a mixture of 1x RIPA Lysis Buffer (Upstate® Cell Signaling Solutions) and protease inhibitors. The samples were processed using a Sample Grinding Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol.

The protocol used for western immunoblot analysis included a Bradford protein assay (BioRad, Hercules, CA), which was performed in duplicate to determine protein concentrations for each sample. Equal amounts of protein extract were electrophoresed using Criterion SDS-PAGE (Bio-Rad) and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in wash buffer (containing 1 x BioRad Tris-Buffered Saline with Tween 20) and incubated overnight with appropriate primary antibodies. Following washes, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Membranes were washed one final time and subjected to chemiluminesence with SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Woburn, MA). Molecular weight lane markers were used to ensure that the band was at the size indicated by the manufacturer of each antibody. The specificity of these antibodies has been validated in our lab using appropriate cell lines or blocking peptides. The relative intensity of the protein bands was obtained by autoradiogram and scanned using a VersaDoc 4000 densitometer (BioRad). Quantity One software (BioRad) was used to quantify band intensity. Proteins were blotted and confirmed at least twice for verification.

Immunohistochemical Analysis

Uteri were dissected, formalin-fixed, and blocked in paraffin wax. Five-μm-thick tissue sections were cut from paraffin-embedded tissue blocks and mounted on glass slides. Antigen retrieval was performed by immersing sections in 0.6 *M* citrate buffer and microwaving on high power for 20 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 10 min. Antigens were detected using the Vectastain Elite kit (Vector) according to the manufacturer's instructions. Briefly, sections were blocked in serum for 90 min. Sections were then incubated with a primary antibody for SOD1 (Santa Cruz). Sections were subsequently incubated with corresponding biotin-labeled secondary antibody, followed by peroxidase-labeled avidin complex (Vector). Finally, the sections were developed with DAB and counterstained with hematoxylin. Negative controls were performed by staining slides with no primary antibody exposure. Coded slides were examined under a light microscope. Digital images were captured using a Nikon digital camera and images analyzed using Image J software. Differences in staining localization and intensity were observed under the guidance of a board certified UAB pathologist.

Serum Hormone Levels

Serum levels of 17β–estradiol and progesterone were measured using radio-immunoassays (Diagnostic Systems Laboratories, Webster, TX) as described by the manufacturer. All samples were run in duplicate.

Statistical Analysis

Statistics were performed using Microsoft Excel 2003 software. Student's t-test was used to perform comparative analysis between control (sesame oil) and treatment (TCDD) groups. Statistical significance was defined as a p value of ≤ 0.05.

RESULTS

Body Weights, Uterine Weights, and Serum Hormone Levels

21-Day-old rats exposed prenatally to TCDD had significantly decreased body weight as compared to age-matched control rats (20%; Table **1**). Uterine wet weights were slightly, but not significantly, decreased in TCDD-exposed rats (11%), and the calculated uterine to body weight ratios were not significantly different. At 50 days postpartum, TCDD-exposed rats had slight, but statistically significant, reductions in body weight (8%; Table **1**) compared to controls. Uterine wet weights and uterine to body weight ratios tended to be decreased in the TCDD-exposed rats, but this did not reach statistical significance (11.5 and 3.5% decreases, respectively).

Table 1. Body and Uterine Weights, and Uterine to Body Weight Ratios in 21- and 50-Day-Old Female Rats Exposed Prenatally to TCDD

Animal Group (n)	Body Weight (g)	Uterine Weight (mg)	Uterine: Body Weight (mg/g)
21 day control (10)	54.6 ± 2.0	32.2 ± 2.3	0.59 ± 0.04
21 day TCDD (10)	43.9 ± 3.0[b]	28.5 ± 2.1	0.67 ± 0.06
50 day control (20)	198.9 ± 3.4	338.5 ± 15.2	1.71 ± 0.08
50 day TCDD (20)	183.7 ± 3.5[a]	299.5 ± 13.8	1.65 ± 0.17

Timed pregnant Sprague-Dawley CD female rats were gavaged with 3 μg TCDD/kg body weight or an equivalent volume of sesame oil (controls) on day 15 post-conception. At birth, offspring were cross-fostered to untreated dams (surrogate mothers). Each treatment group contained 30 dams with only one female offspring examined per dam. Values represent means ± SEM. [a] $P < 0.005$ and [b] $P < 0.010$ as compared with age-matched controls.

Circulating 17β-estradiol and progesterone concentrations were found to be similar at 50 days postpartum between control and TCDD-exposed groups. 17β-Estradiol levels were 13.7 ± 2.4 pg/ml serum in control animals and 16.2 ± 3.2 pg/ml serum in TCDD-exposed animals. Progesterone levels were 15.0 ± 2.4 ng/ml serum in control animals and 16.9 ± 3.1 ng/ml serum in TCDD-exposed animals.

Uterine Morphology and Epithelial Cell Height

Dissected uteri from 50-day-old rats, a critical time point post-puberty, were fixed in 10% neutral buffered formalin and 5 μm sections were cut onto slides. Each slide contained one uterus from a control (sesame oil) rat and one uterus from a TCDD-treated rat. The slides were stained *via* a standard H&E method. Under the guidance of a UAB board-certified pathologist (Dr. Isam Eltoum), uterine morphology was compared between sesame oil and TCDD-treated rats. As shown in Fig. (**1**), there were obvious differences between the groups in terms of uterine morphology.

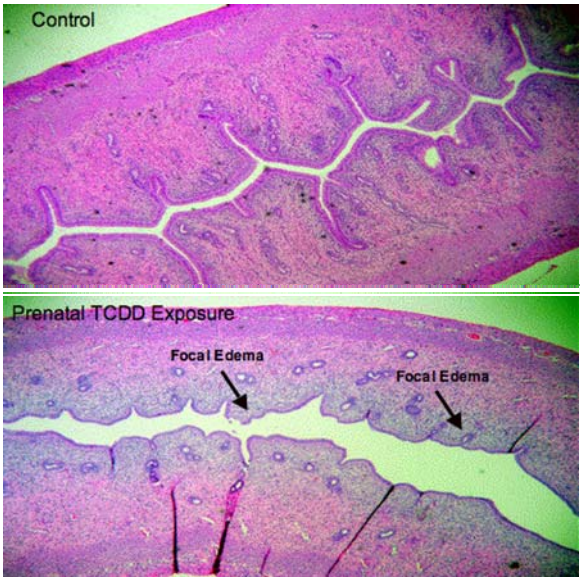

Fig. (1). H&E stained uteri at 50 days postpartum. The top panel represents a uterine section of a sesame oil-treated control rat, while the bottom panel represents a uterine section of a rat exposed prenatally to TCDD. The black arrows point to the pale staining area adjacent to the luminal epithelium that indicates focal edema.

The TCDD-exposed uteri demonstrated a visual decrease in uterine branching. The uterine epithelial cells were atrophic in appearance compared to the controls. To further investigate uterine epithelial atrophy and cell size, luminal epithelial cell height was measured. There was a statistically significant 25% decrease in luminal epithelial cell height in the uteri of the TCDD-treated rats compared to those rats treated with sesame oil (39.8 ± 0.6 μm versus 30.3 ± 0.7 μm in control versus TCDD-exposed rats). This confirmed morphological changes observed in the H&E stained uteri. In several of the TCDD-treated uteri (5/7), focal edema was observed (black arrows Fig. **1**), adjacent to the luminal epithelial cells. There was also an observed increase in the diffusion of eosinophil leucocytes in the stroma and muscle layers of the uteri prenatally exposed to TCDD.

Cell Proliferation

Cell proliferation was measured *via* Ki67 immunohistochemistry in the uterus. In the glandular and luminal epithelia of 21-day-old rats exposed prenatally to TCDD compared to controls, cell proliferation was significantly decreased by four- and two-fold, respectively (Table **2**) and (Fig. **2**). Conversely, in 50-day-old rats exposed prenatally to TCDD, cell proliferation was significantly increased (two-fold) in the luminal epithelium but there was no significant change of cell proliferation in the glandular epithelium of these sexually mature animals.

Apoptosis

At 21 days postpartum, the percentage of uterine cells undergoing apoptosis was not significantly changed with

prenatal exposure to TCDD in the glandular and luminal epithelia (Table **3**) and (Fig. **3**). Conversely, in uteri of 50-day-old rats, an age affected by the sex steroids associated with puberty and sexual maturity, apoptosis was significantly decreased by ten- and four-fold in the glandular and luminal epithelia, respectively following prenatal TCDD exposure.

Table 2. Cell Proliferation Index in Uteri of 21- and 50-Day-Old Female Rats Exposed Prenatally to TCDD

Animal Group (n)	Glandular Epithelium	Luminal Epithelium
21 day control (6)	5.18 ± 1.27	6.10 ± 0.91
21 day TCDD (6)	1.21 ± 0.52[a]	3.31 ± 0.42[a]
50 day control (6)	5.40 ± 1.03	7.83 ± 1.38
50 day TCDD (6)	5.91 ± 1.91	14.47 ± 3.27[b]

Timed pregnant Sprague-Dawley CD female rats were gavaged with 3 µg TCDD/kg body weight or an equivalent volume of sesame oil (controls) on day 15 post-conception. At birth, offspring were cross-fostered to untreated dams (surrogate mothers). Values represent means ± SEM. [a] $P < 0.01$ and [b] $P < 0.05$ as compared with age-matched controls.

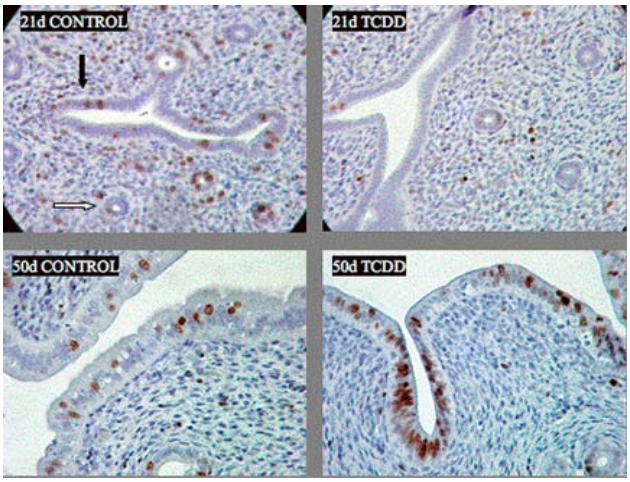

Fig. (2). Cell proliferation as measured by Ki-67 expression in uteri of 21-day-old and 50-day-old rats exposed prenatally to TCDD or sesame oil. DAB staining (brown) for Ki67 was counted as indicator of cell proliferation in the glandular (white arrow) and luminal (black arrow) epithelia. Pictures were taken at 400x magnification.

Table 3. Apoptosis Index in Uteri of 21- and 50-Day-Old Female Rats Exposed Prenatally to TCDD

Animal Group (n)	Glandular Epithelium	Luminal Epithelium
21 day control (6)	10.00 ± 1.91	3.20 ± 0.46
21 day TCDD (6)	8.70 ± 1.55	4.10 ± 0.67
50 day control (6)	40.30 ± 3.50	43.40 ± 2.50
50 day TCDD (6)	4.00 ± 2.00[a]	10.80 ± 2.50[a]

Timed pregnant Sprague-Dawley CD female rats were gavaged with 3 µg TCDD/kg body weight or an equivalent volume of sesame oil (controls) on day 15 post-conception. At birth, offspring were cross-fostered to untreated dams (surrogate mothers). Values represent means ± SEM. [a] $P < 0.001$ as compared with age-matched controls.

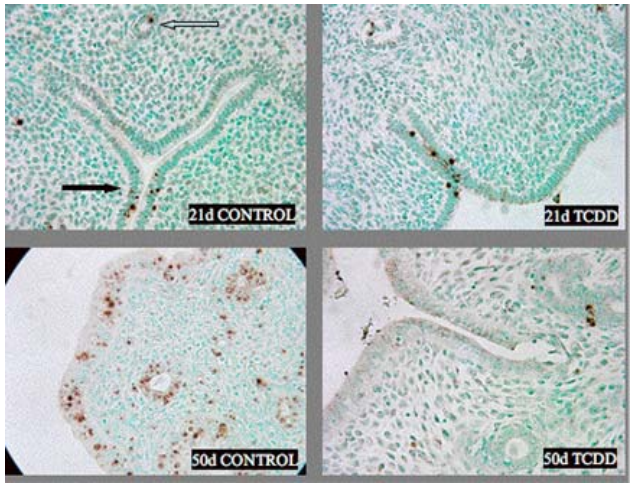

Fig. (3). Apoptosis in uteri of 21-day-old and 50-day-old rats exposed prenatally to TCDD or sesame oil. DAB staining (brown) was counted as indicator of apoptosis in the glandular (white arrow) and luminal (black arrow) epithelia. Pictures were taken at 400x magnification.

EGF Pathway Modulation

In the uteri of 50-day-old rats, we observed modulation of EGFR protein expression. EGFR was significantly up-regulated by two-fold in uteri of 50-day-old rats exposed prenatally to TCDD (Fig. **4**). This increase in uterine EGFR in the TCDD-treated rats coincides temporally with increased uterine epithelial proliferation and decreased apoptosis. EGFR protein expression in the uterus was not altered at 21 days postpartum following prenatal TCDD exposure (data not shown).

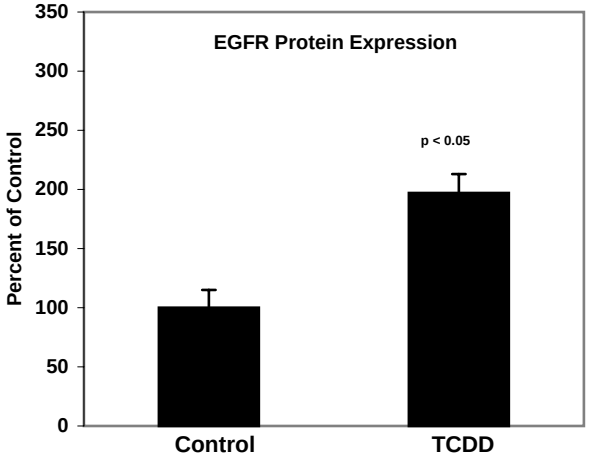

Fig. (4). EGFR protein expression in uteri of 50-day-old rats exposed prenatally to TCDD or sesame oil. EGFR protein was assayed *via* western blot analysis with each group containing a minimum of six samples. Values represent mean ± SEM as a percent of the Control group. A p value < 0.05 was considered statistically significant.

SOD1 Expression and Localization

SOD1 is one of the primary scavengers of reactive oxygen species. TCDD has been shown to increase the amount of oxygen radicals, including superoxide anions. Here we show that protein levels of SOD1 were found to be signifi-

cantly down-regulated in uteri of TCDD exposed rats by 20% (Fig. **5**). Protein expression and localization were visualized in the uteri of control and TCDD-treated glands by IHC. At 50 days postpartum, the uteri from TCDD-exposed rats showed a significant, visual reduction in the staining for SOD1 (Fig. **6**), confirming the protein expression results obtained by western blot analysis. SOD1 staining was primarily observed within the cytoplasm of both glandular and luminal uterine epithelial cells.

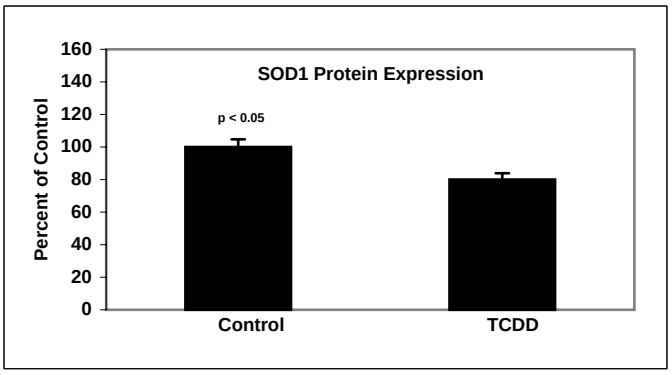

Fig. (5). SOD1 protein expression in uteri of 50-day-old rats exposed prenatally to TCDD or sesame oil. SOD1 protein was assayed *via* western blot analysis with each group containing a minimum of six samples. Values represent mean density ± SEM as a percent of the Control group. $p < 0.05$ was considered statistically significant.

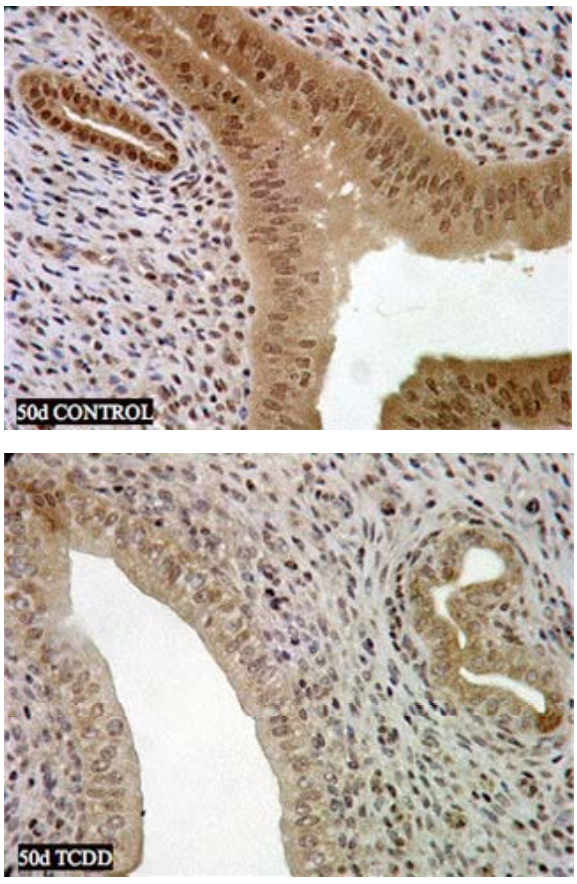

Fig. (6). Immunohistochemical stain for SOD1 in a uterine section of 50-day-old rats exposed prenatally to TCDD or sesame oil.

Sex Steroid Receptors

In the uteri of 21- and 50-day-old rats exposed prenatally to TCDD, we measured the protein levels of the estrogen receptors α and β and the progesterone receptors A and B by western blot analysis. We observed no changes in the protein levels of these steroid receptors at 21 or 50 days postpartum in the TCDD-treated rats compared to the control rats (data not shown).

DISCUSSION

TCDD has been shown to cause detrimental alterations in multiple, female reproductive organs. Previously, we showed that *in utero* TCDD exposure could enhance susceptibility for mammary carcinogenesis [9]. Reports have also demonstrated that the uterus is a susceptible organ to TCDD exposure, and can cause uterine carcinomas [4]. In this report, we investigated mechanisms by which TCDD could possibly increase uterine susceptibility to disease, especially cancer.

Body Weights, Uterine Weights, and Serum Hormone Levels

Our finding that prenatal TCDD exposure resulted in decreased body weights at days 21 and 50 postpartum are consistent with our previous results and other reports that TCDD treatment (prenatal, perinatal, or prepubertal) resulted in decreased body weights in rats at doses that range from 1 μg TCDD/kg body weight to 75 μg TCDD/kg body weight [9,20-22]. Decreased appetite has been implicated to be a contributing factor for the commonly seen decrease in body weights in TCDD-exposed rats [23-25].

In this study, uterine wet weights tended to be lower in the TCDD-exposed group at both 21 and 50 days postpartum, but the differences were not statistically significant. This is consistent with a study done by Gray *et al.* who administered 1 μg TCDD/kg body weight treatment to pregnant female rats on day 15 of gestation and showed a slight, but not significant, decrease in uterine weights in rats [26]. Also, they showed that ovarian weight and the weight of the female reproductive tract (vagina, cervix, and uterus) were slightly, but not significantly, reduced. The downward trend in uterine weights may indicate a growth suppressive effect on the uterus, similar to what occurs in the mammary gland, which has been documented in several studies [3,8,9]. These reports showed that animals treated with TCDD have delayed maturation and growth of the mammary gland, and the present study may indicate a similar effect for the rat uterus. We have previously shown that prenatal TCDD exposure also results in reduced liver weights in rats at 50 days postpartum [9].

In the present study, no differences in serum estrogen (17β–estradiol) and progesterone concentrations were detected at 50 days postpartum. Chaffin *et al.* have reported a significant reduction of circulating estrogen in prepubertal rats that were exposed to TCDD *in utero* [27]. This decrease in circulating estrogen by TCDD has been associated with the stimulation of 17β-estradiol hydroxylase [28]. Because our study looked at circulating hormones postpubertally, endogenous estrogen production may compensate for any reduction that *in utero* TCDD exposure may cause. Others have shown that TCDD exposure does not affect circulating

estrogens in CD1 mice, thus there may exist strain- and species-dependent effects [29].

Changes in Uterine Morphology

Several alterations in uterine morphology were observed in the TCDD-exposed uteri at 50 days postpartum. The uteri of the TCDD-exposed rats appeared atrophic and with decreased luminal branching compared to the age-matched controls (sesame oil). A 25% reduction in luminal epithelial cell height confirmed that *in utero* TCDD exposure had a growth suppressive effect in the uterus. This delay in maturation has been reported in the mammary glands of rats that were exposed perinatally to TCDD [3,8,9,]. There were also signs of focal edema adjacent to the luminal epithelial cells in the uteri of TCDD-exposed rats, along with observations of increased diffusion of eosinophil leukocytes in the stroma and muscle layers. These white blood cells are often associated with the combating of infection and are also associated with inflammation. Inflammation may be associated with endometrial cancer and cell transformation [30]. An infiltration of eosinophil leukocytes has been reported in the uterus of rats after exposure to M. *spicata*, which causes lipid peroxidation and uterine damage [31].

Changes in Cell Proliferation

Using IHC, uterine sections were measured for Ki67 antigen expression as an indicator of cell proliferation. At 21 days postpartum, cell proliferation was down-regulated in both the glandular and luminal epithelial cells of animals exposed prenatally to TCDD. The difference represents approximately a four-fold decrease in cell proliferation in the glandular epithelium and a two-fold decrease in cell proliferation in the luminal epithelium of the 21-day-old offspring. In contrast, the uterine epithelial cells of TCDD-exposed rats were significantly more proliferative at 50 days postpartum than the age-matched control rats, despite an atrophic appearance. Cell proliferation was shown to be up-regulated by two-fold in the luminal epithelium of 50-day-old animals exposed prenatally to TCDD. In the glandular epithelium, there was a slight, although not statistically significant, increase in cell proliferation. While both uterine epithelial compartments showed increased cell proliferation at 50 days postpartum, the luminal epithelium responded more strongly to prenatal TCDD exposure. Interestingly, the glandular epithelial cells have a greater apoptotic response to prenatal TCDD exposure. Thus, the epithelial compartments of the uterus may respond differently to TCDD exposure. This uterine environment of atrophic endometrium while maintaining proliferative capacity has also been observed in postmenopausal women [32]. In that study, 84 postmenopausal women asymptomatic for endometrial disease were examined. All of the uteri were atrophic although 50% retained some proliferative capacity as measured by Ki67. Thus, the observed increase in uterine cell proliferation may make the uterus more disposed to disease, including cancer.

Thus, before the onset of puberty and the hormonal signaling pathways that are associated, the uteri of TCDD-exposed rats are less proliferative. This could play a role in the less mature uterus that is observed at 50 days postpartum. In a study examining the mouse uterus, Buchanan *et al.* found that the anti-proliferative effects of TCDD on uterine epithelia appeared to be mediated indirectly through the Aryl hydrocarbon receptor (AhR) in the stroma. The authors suggested that TCDD inhibits uterine epithelial responses to 17β-estradiol by acting through the stromal AhR [33].

Uterine Apoptosis

We measured uterine epithelial apoptosis by an indirect TUNEL method, and calculated apoptotic indices for both the glandular and luminal epithelia of 21-day-old and 50-day-old animals treated prenatally with TCDD. At 21 days, apoptosis was unaffected by the prenatal TCDD treatment in both the glandular and luminal epithelia. This could be related to the fact that 21-day-old rats have not reached puberty, and thus are not under the influence of estrogens, which are known to play a role in uterine apoptosis. At 50 days, apoptosis was drastically down-regulated in both the glandular and luminal epithelia of rats exposed prenatally to TCDD, even with all rats being in the estrous phase of the cycle. The difference represents a ten-fold decrease in apoptosis in the glandular epithelium and a four-fold decrease in apoptosis in the luminal epithelium. The fact that apoptosis was not altered at day 21 postpartum, but was at day 50, argues against residual TCDD concentrations being directly responsible for apoptotic events. Rather, imprinting mechanisms and the response to puberty may play a role in causing effects early in development which are not displayed until later in life, and in the case of the present study, after puberty.

The findings at 50 days of age have remarkable implications: the luminal epithelium was found to have a two-fold increase in cell proliferation and a four-fold decrease in apoptosis. With these two factors combined, the luminal epithelium of animals treated prenatally with TCDD may be highly susceptible to uterine disease later in life. Our study suggests that TCDD may promote disease in the uterus by increasing cell proliferation and decreasing apoptosis.

Sex Steroid Receptors

Uterine growth and cancer are often associated with altered regulation of ER and PR levels. Estrogen and progesterone have the ability to promote cell proliferation in the breast and uterus. It could be hypothesized that the increase in cell proliferation in the uterus, as was found in the present study at 50 days postpartum, could be caused by an increase in the activity of ER and/or PR. However, no significant change in protein expression of either of the estrogen receptors or progesterone receptors at 21 or 50 days in the TCDD-exposed animals was observed (data not shown). There were also no significant difference in the circulating levels of 17β-estradiol and progesterone. We suspect that the proliferative effects measured at 50 days are not tied to differing levels of these sex steroid receptors, but rather to other vital uterine growth factors.

EGF Signaling

In the present study, there was a significant increase in protein expression of EGFR at 50 days, a known player associated with cell proliferation in the uterus [11]. As mentioned above, studies in our lab have associated uterine proliferation with increased levels of EGFR [15,16]. Also, it has

been shown that inactivation of EGFR by selective inhibitors significantly decreases levels of extracellular regulating kinase 2 (an important mediator of signal transduction by EGFR) activation, c-fos (an important oncoprotein for signal transduction, cell proliferation, and differentiation) RNA expression, and cell proliferation [34]. Thus, up-regulation of EGFR might help to promote cell proliferation, as was found in the present study in the luminal epithelium of 50 day animals treated prenatally with TCDD. Previous reports have noted that TCDD exposure results in an increased expression of TGF-α, a known ligand for the EGFR, following TCDD exposure [13].

SOD1: Protein Expression and Localization

In the present study, we found SOD1 protein levels in uteri of TCDD-exposed animals at 50 days postpartum to be significantly down-regulated as compared to controls. The TCDD-exposed rats had approximately 20% less SOD1 compared to controls. This finding is important as SOD1 neutralizes supercharged oxygen molecules. Superoxide radicals, which are by-products of normal and pathologic cell processes, can damage cells if their levels are not tightly controlled by SOD enzymes. TCDD exposure has been associated with an increase in superoxide anion levels [reviewed in 12]. The same review also noted that TCDD has also been shown to increase levels of prostaglandin synthesis and numerous cytokines that are associated with free radical production. The significant down-regulation of SOD1 that we observed by TCDD-treatment may increase the potential for free radical damage. These radicals can bind to DNA, proteins, and lipids and cause permanent loss of structure and play a significant role in initiation and promotion mechanisms. The reduction in the levels of SOD1 indicate that TCDD-exposed animals may be more susceptible to cellular structure damage brought on by reactive oxygen radicals that cannot be converted as readily to a more benign species by SOD1 and subsequent enzymes such as catalase and glutathione peroxidase. It has been shown previously that TCDD treatment increases mRNA levels for tumor necrosis factor-alpha (TNF-α) in human uterine endometrial adenocarcinoma RL95-2 cells [35]. TNF-α is a potent pyrogen that can cause inflammation by stimulation of interleukin-1 secretion. Thus an increase in TNF-α, free radicals and a diminished amount of SOD1 enzyme could create a uterine environment that is more susceptible to disease.

The protein expression and localization of SOD1 were also investigated by immunohistochemical techniques. The results with respect to expression level confirm the results from the immuoblot analysis. There was a visual reduction of SOD1 protein levels (Fig. **6**) in the uteri of rats treated prenatally with TCDD. As one would expect, SOD1 was located mainly to the cytoplasmic portions of epithelial cells and blood vessels in both control and TCDD-exposed rat uteri. There was also evidence of some nuclear staining, although less than in the cytoplasm. These localizations would be expected based on reports looking at SOD1 localization in multiple species and cell types [36-39]. This confirmation of a decreased amount of SOD1 confirms that prenatal treatment with TCDD may leave the uterus less efficient at the removal of free radicals, and thus more susceptible to damage and subsequent disease.

CONCLUSIONS

It has been well established that prenatal exposure to TCDD can affect later susceptibility to mammary and uterine disease although the mechanisms for these effects are not well understood. In this report, we show that prenatal exposure to TCDD can affect uterine growth and morphology, cell proliferation, apoptosis, and the expression of several key proteins, known to play a role in uterine growth and uterine diseases, including cancer. We recognize that the model system in this report was not ideal for uterine tumorigenesis, but feel that the changes we observed could be predictive of a uterine environment that is more susceptible to disease. With prenatal TCDD exposure, there were several alterations in the uterus that could create an environment more favorable for uterine disease and carcinogen insult, including: (1) up-regulating cell proliferation in the luminal epithelium of the uterus; (2) down-regulating apoptosis in both the glandular and luminal epithelia, which could allow damaged or tumorigenic cells to remain viable; (3) up-regulating the protein expression of key growth factors such as EGFR, which could allow more ligand to bind to the receptor and cause signal transduction in growth factor signaling pathways; (4) down-regulation of biomarkers that serve to maintain the integrity of cellular infrastructure and protect against free radical damage, such as SOD1. Later in life, the rats exposed prenatally to TCDD may be at an increased risk for uterine disease such as endometriosis or cancer due to changes in sex steroid and growth factor signaling. Because it is unlikely that high concentrations of TCDD remain in the uteri of 50 day offspring whose mothers were exposed to TCDD, we conclude that gestational TCDD causes permanent developmental alterations to uterine proteins that are manifested later in life, especially after the signaling events of puberty. We strongly believe, based on the results of this study, that prenatal exposure to TCDD can increase uterine disease susceptibility later in life.

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