Toxicity of Chronic Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin in DiethylNitrosamine-Initiated Ovariectomized Rats Implanted with Subcutaneous 17 β-Estradiol Pellets

Michael E. Wyde,*† John Seely,‡ George W. Lucier,† and Nigel J. Walker†‡

*Curriculum in Toxicology, University of North Carolina, Chapel Hill, North Carolina; †National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709; and ‡Pathco Inc., Research Triangle Park, North Carolina

Received July 23, 1999; accepted November 22, 1999

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent hepatocarcinogen in female but not in male rats. Several lines of evidence suggest a key role of ovarian hormones, presumably estrogen, in the mechanism of TCDD-induced hepatocarcinogenesis. The aim of this current study was to determine the toxicity of co-treatment with TCDD and 17 β-estradiol and assess the efficacy of 90-day subcutaneous constant release 17 β-estradiol pellets. Ovariectomized (OVX) female Sprague-Dawley rats were initiated with diethylnitrosamine (DEN) and treated with TCDD for 20 or 30 weeks in the presence and absence of 17 β-estradiol. TCDD concentrations were equivalent in livers of TCDD-treated sham operated and OVX rats following 20 weeks of treatment. Following 30 weeks of TCDD treatment, liver TCDD concentrations were higher in OVX rats than in intact rats. TCDD concentrations in livers of TCDD-treated OVX rats receiving supplemental 17 β-estradiol were similar to intact rats following either 20 or 30 weeks of treatment. Mean hepatic background TCDD concentrations in untreated rats were 2-fold higher in intact rats compared to OVX rats, regardless of 17 β-estradiol exposure following 20, but not 30 weeks of treatment. Serum indicators of hepatocellular and hepatobiliary toxicity indicated transient hepatotoxicity in TCDD-treated OVX rats receiving 17 β-estradiol. Histopathological alterations indicated hepatotoxicity induced by exposure to TCDD following either 20 or 30 weeks of exposure. No excess hepatotoxicity was associated with 17 β-estradiol-supplementation in TCDD-exposed OVX female Sprague-Dawley rats. Serum 17 β-estradiol concentrations were not constant and resulted in supra-physiological levels that decreased over time, resulting in target physiological serum 17 β-estradiol concentrations following several weeks of release. Treatment with 17 β-estradiol resulted in uterine weights and total body weights comparable to sham-operated female rats. These data confirm the efficacy of supplemental subcutaneous 17 β-estradiol pellets on the induction of estrogenic responses in TCDD-treated rats and indicate no increased hepatotoxicity associated with 17 β-estradiol exposure in TCDD-treated rats.

Key Words: 2,3,7,8-tetrachlorodibenzo-p-dioxin; 17 β-estradiol; hepatocarcinogenesis; clinical chemistry; subcutaneous pellets.

1 To whom correspondence should be addressed at the Laboratory of Computational Biology and Risk Analysis, N.I.E.H.S., MD D4-01, PO Box 12233, Research Triangle Park, NC 27709. Fax: (919) 541-4704. E-mail: walker3@niehs.nih.gov.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent congener of the polyhalogenated aromatic hydrocarbons (Poland and Knutson, 1982) and a persistent and widely dispersed environmental contaminant. Exposure to TCDD results in a broad spectrum of toxicity and biochemical alterations in both humans and experimental animals (Bertazzi et al., 1998; Huff et al., 1994; Kociba and Schwetz, 1982; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982). TCDD is believed to exert its toxic responses via high-affinity binding to the aryl hydrocarbon receptor (AhR) (Schmidt and Bradfield, 1996). An active TCDD-AhR complex acts as a nuclear transcription factor regulating the expression of numerous dioxin-responsive genes (Sutter and Greenlee, 1992; Swanson and Bradfield, 1993) including several cytochromes P450 (Whitlock et al., 1997). However, the role of these and other biochemical alterations in the induction of tumorigenesis by TCDD has not been established.

TCDD is a multi-site rodent carcinogen in both sexes (Kociba et al., 1978; National Toxicology Program, 1982; Huff et al., 1994); however, it is not directly genotoxic (Kociba, 1984; Poland and Glover, 1979; Wassom et al., 1977). In the 2-stage model for carcinogenesis, TCDD is a potent tumor promoter in rodent skin and liver (Lucier et al., 1991; Pitot et al., 1980; Poland et al., 1982). Currently, the mechanism for the induction of liver tumors by TCDD in female rats is unknown. Several lines of evidence support a role for ovarian hormones in the mechanism of TCDD-induced hepatocarcinogenesis. Chronic exposure to TCDD induces liver tumors in female but not male rats (Kociba et al., 1978; National Toxicology Program, 1982). In 2-stage initiation-promotion models, hepatic tumor incidence is lower in TCDD-treated, ovariectomized (OVX) female Sprague-Dawley rats than in TCDD-treated, sham-operated rats (Clark et al., 1991). Increases in preneoplastic altered hepatocellular foci and 8-hydroxydeoxyguanosine oxidative DNA adducts are greater in sham-operated rats than in OVX female Sprague-Dawley rats (Lucier et al., 1991; Tritscher et al., 1996). TCDD-induced cell proliferation is also dependent upon ovarian hormones (Lucier et al., 1991). It is hypothesized that estrogen is involved in the mechanism of TCDD-induced hepatocarcinogenesis by the enzyme-medi-
ated metabolic activation of estradiol to directly or indirectly acting reactive intermediates (Yager and Liehr, 1996) or through alteration of cell-growth pathways (Sewell et al., 1993). To test this hypothesis, a chronic initiation-promotion study in OVX TCDD-treated female rats receiving supplemental 17β-estradiol was conducted to evaluate estrogen modulation of TCDD-induced tumorigenesis. TCDD induces cytochrome P450 isozymes involved in the metabolism of estradiol (Graham et al., 1988; Martucci and Fishman, 1993; Ryan et al., 1984). TCDD also inhibits 17β-estradiol-induced responses in female Sprague-Dawley rats (Safe, 1995). Due to these effects of TCDD, it is necessary to evaluate the effect of chronic co-exposure to TCDD and estradiol. In this paper we describe the efficacy of supplemental treatment with subcutaneous 90-day release 17β-estradiol pellets and determine if there is any treatment-related toxicity resulting from co-treatment of TCDD and 17β-estradiol in DEN-initiated OVX female Sprague-Dawley rats. Further, we describe the effects of 17β-estradiol on hepatic TCDD dosimetry and of 17β-estradiol on estradiol-dependent responses in vivo, and we assess the efficacy of subcutaneous constant-release 17β-estradiol pellets and the hepatotoxicity associated with co-treatment with TCDD and 17β-estradiol in DEN-initiated female Sprague-Dawley rats.

**MATERIALS AND METHODS**

**Animals.** Female Sprague-Dawley rats were ovariectomized or sham operated at 8 weeks of age by Charles River Labs (Raleigh, NC). Animals were housed 3 per cage and received food and water ad libitum under conditions of controlled temperature (70 ± 0.5°F), humidity (50 ± 5%), and light (12-h light/dark cycle). OVX and sham-operated (intact) rats were initiated with a single dose of 175 mg diethylnitrosamine (DEN)/kg of body weight (bw) at 10 weeks-of-age. At 11 weeks-of-age, OVX rats were implanted with 90-day release subcutaneous pellets containing 0.18 mg of 17β-estradiol per pellet (Innovative Research of America, Sarasota, FL). Intact rats and OVX rats not treated with 17β-estradiol were implanted with matching placebo pellets containing a mixture of cholesterol, cellulose, alpha-lacto-cue, calcium phosphate, calcium and magnesium stearate, and stearic acid. New estrogen or placebo pellets were implanted on the ninety-first day following implantation. At 12 weeks of age, animals began treatment with weekly gavage doses of corn oil or 700 ng/kg TCDD in corn oil to approximate a daily average dose of 100 ng/kg, as a promoting agent, for either 20 or 30 weeks. Osmotic pumps (Alzet 2ML1 10 μl/h delivery; Alzet Corp., Palo Alto, CA) containing 30 mg/ml 5-bromo-2'-deoxyuridine in saline were implanted 7 days prior to necropsy. Terminal serum was obtained, under CO₂ anesthesia, from cardiac blood. Animals were killed by asphyxiation with CO₂. Tissues were removed, weighed, sectioned, and fixed in parafomaldehyde or frozen in liquid nitrogen.

**Assessment of serum 17β-estradiol.** Three additional OVX animals per group were treated in order to assess 17β-estradiol release from the subcutaneous pellets. Serum was collected by orbital sinus puncture 14, 50, and 85 days following implantation. 17β-Estradiol levels of terminal and periodic serum were measured by radioimmunoassay (Estradiol Double Antibody; Diagnostic Products Corp., Los Angeles, CA).

**Dosimetry of TCDD.** Livers of control and treated rats were analyzed by gas chromatography-mass spectrometry as previously described (Lucier et al., 1991) to quantitate levels of TCDD.

**Histopathology.** Liver tissue was fixed in 4% paraformaldehyde and paraffin-embedded. Histological evaluation was performed on 5-μm sections stained with hematoxylin and eosin.

**Clinical chemistry.** Serum activities of alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH), alkaline phosphatase (ALP) and 5'-nucleotidase (5ND), and the concentration of total bile acids were determined on a Monarch 2000 chemistry analyzer (Instrumentation Laboratory, Inc., Lexington, MA). ALT and ALP activities were measured using reagents obtained from the instrument manufacturer. SDH and 5ND activities and bile acid concentrations were measured using reagents obtained from Sigma Diagnostics (St. Louis, MO).

**Statistics.** All significant differences except for uterine weight and hepatic TCDD concentrations were determined by ANOVA. Subsequent complex comparisons were determined by Fisher’s least significant difference. Log-transformed and non-transformed uterine weights were determined not normally distributed by Bartlett’s test for homogeneity of variance. Therefore, significant differences for uterine weights were determined by the Mann-Whitney U test. Significant differences for hepatic TCDD concentrations were determined by 2-tailed Student’s t-test. Significant differences were reported at p < 0.05.

**RESULTS**

**TCDD liver dosimetry.** Mean hepatic TCDD concentrations in TCDD-treated intact and OVX rats receiving placebo pellets were 15,208 and 17,946 ppt wet weight, respectively following 20 weeks of TCDD exposure (Table 1). Ovariectomy did not significantly affect hepatic TCDD concentrations. TCDD concentrations in livers of OVX rats co-treated with 17β-estradiol and TCDD were not significantly different from OVX rats receiving only TCDD. However, TCDD concentrations in the livers of OVX rats receiving corn oil, regardless of 17β-estradiol treatment, were 2-fold higher than intact rats receiving corn oil alone. In animals not treated with TCDD, average values were 25 ppt wet weight in livers of intact rats compared to 60 in OVX rats and 66 in OVX rats receiving 17β-estradiol.

Following 30 weeks of TCDD treatment, mean hepatic TCDD concentrations in TCDD-treated intact OVX rats were significantly higher than both TCDD-treated intact rats and TCDD-treated OVX rats receiving supplemental 17β-estradiol. Mean hepatic TCDD concentrations were 12,963 ppt wet weight in livers of intact rats and 13,075 ppt wet weight in livers of OVX rats receiving 17β-estradiol compared to 16,769 ppt wet weight in livers of OVX rats. There were no significant differences in hepatic TCDD concentrations in rats receiving corn oil, regardless of ovariectomy or 17β-estradiol treatment.

**Assessment of serum hormones.** Treatment with 17β-estradiol-releasing pellets resulted in supra-physiological levels of serum estradiol by 14 days post-implantation (Fig. 1). Physiological levels of serum 17β-estradiol levels in rats ranges from 1.2–88 pg/ml depending on the phase of the estrous cycle (Overpeck et al., 1978). Serum 17β-estradiol concentrations were not constant and decreased over time, resulting in target physiological serum 17β-estradiol concentrations several weeks after implantation.

As expected, ovariectomy significantly decreased serum 17β-estradiol concentrations in female rats following 20 weeks of promotion (Table 1). Following 30 weeks of promotion, serum estradiol concentrations were lower in OVX rats than in intact...
rats. Serum estradiol concentrations were significantly higher in OVX rats receiving supplemental 17\(\beta\)-estradiol than in OVX rats receiving placebo pellets. The observed increase in serum estradiol concentrations in OVX rats were seen in both TCDD-treated and control rats at both time points. Serum estradiol concentrations were significantly lower in OVX rats receiving 17\(\beta\)-estradiol-supplemented OVX rats as compared to those receiving placebo pellets. In rats receiving 17\(\beta\)-estradiol-supplemented OVX rats following 30 weeks of exposure to TCDD on serum estradiol concentration regardless of ovariectomy or 17\(\beta\)-estradiol treatment.

The uterine wet weight (an estrogen-responsive tissue) was significantly higher in OVX rats receiving 17\(\beta\)-estradiol treatment when compared to those receiving placebo pellets. (Table 1). Uterine wet weights of OVX rats receiving supplemental 17\(\beta\)-estradiol were closer to those of intact rats. There were no observed effects of chronic exposure to TCDD on 17\(\beta\)-estradiol induced uterine wet weight.

**Body weight.** The body weights of OVX rats were significantly higher than both intact rats and OVX rats receiving supplemental 17\(\beta\)-estradiol, following both 20 and 30 weeks of treatment (Table 1). Similar results were observed for intact and 17\(\beta\)-estradiol-supplemented OVX rats following 30 weeks of treatment but not OVX rats receiving placebo pellets.

Alterations in the rate of body weight gain were also observed in rats receiving 17\(\beta\)-estradiol pellet implants (Fig. 2). The rate of body weight gain for OVX rats receiving 17\(\beta\)-estradiol were comparable to intact rats, but considerably lower than OVX rats receiving placebo pellets. In rats receiving 17\(\beta\)-estradiol, body weights decreased dramatically immediately following pellet implants at weeks 1 and 12 (Fig. 2).

The body weights of TCDD-treated intact, OVX, and 17\(\beta\)-estradiol-supplemented OVX rats were comparatively lower than the corresponding corn-oil controls, following 20 weeks of exposure to TCDD on serum estradiol concentration regardless of ovariectomy or 17\(\beta\)-estradiol treatment.

**FIG. 1.** Changes in serum 17\(\beta\)-estradiol levels in rats implanted with 90-day slow release pellets. Ovariectomized female Sprague-Dawley rats were treated with corn oil or an average daily dose of 100 ng/kg TCDD and/or 0.18 mg/pellet 17\(\beta\)-estradiol to approximate daily doses of 2 \(\mu\)g 17\(\beta\)-estradiol. Serum was obtained by orbital puncture 14, 50, and 85 days after pellet implantation and serum estradiol levels were determined by double antibody radioimmunoassay. Mean \(\pm\) standard deviation; \(n = 3\) per group.

<table>
<thead>
<tr>
<th>Dose group</th>
<th>17(\beta)-estradiol (mg/pellet)</th>
<th>Body wt (g)</th>
<th>Relative liver wt (g/100g bw)</th>
<th>Relative uterine wt (g/100g bw)</th>
<th>Serum estradiol (pg/ml)</th>
<th>Liver TCDD (ppt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 Weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact control</td>
<td>0</td>
<td>341 (\pm) 24</td>
<td>3.7 (\pm) 0.4</td>
<td>0.21 (\pm) 0.07</td>
<td>41 (\pm) 13</td>
<td>25 (\pm) 12</td>
</tr>
<tr>
<td>Intact TCDD</td>
<td>0</td>
<td>315 (\pm) 30</td>
<td>3.9 (\pm) 0.3</td>
<td>0.19 (\pm) 0.05</td>
<td>27 (\pm) 11*</td>
<td>15208 (\pm) 3775</td>
</tr>
<tr>
<td>OVX control</td>
<td>0.18</td>
<td>366 (\pm) 51</td>
<td>3.4 (\pm) 0.2</td>
<td>0.16 (\pm) 0.04*</td>
<td>37 (\pm) 10*</td>
<td>66 (\pm) 30*</td>
</tr>
<tr>
<td>OVX TCDD</td>
<td>0.18</td>
<td>322 (\pm) 41</td>
<td>4.2 (\pm) 0.5</td>
<td>0.21 (\pm) 0.03*</td>
<td>49 (\pm) 13*</td>
<td>15343 (\pm) 3708</td>
</tr>
<tr>
<td>30 Weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact control</td>
<td>0</td>
<td>385 (\pm) 75</td>
<td>3.6 (\pm) 0.6</td>
<td>0.21 (\pm) 0.08</td>
<td>36 (\pm) 21</td>
<td>20 (\pm) 8</td>
</tr>
<tr>
<td>Intact TCDD</td>
<td>0</td>
<td>340 (\pm) 36</td>
<td>4.0 (\pm) 0.2*</td>
<td>0.21 (\pm) 0.03</td>
<td>41 (\pm) 22</td>
<td>12963 (\pm) 1790</td>
</tr>
<tr>
<td>OVX control</td>
<td>0.18</td>
<td>355 (\pm) 52</td>
<td>3.7 (\pm) 0.2</td>
<td>0.25 (\pm) 0.02*</td>
<td>68 (\pm) 42*</td>
<td>38 (\pm) 12*</td>
</tr>
<tr>
<td>OVX TCDD</td>
<td>0.18</td>
<td>342 (\pm) 59</td>
<td>4.4 (\pm) 0.3*</td>
<td>0.26 (\pm) 0.05*</td>
<td>50 (\pm) 22*</td>
<td>13075 (\pm) 2070</td>
</tr>
</tbody>
</table>

* Significantly different from intact control, \(p < 0.05\).
* Significantly different from intact animals, \(p < 0.05\).
* Significantly different from OVX control with corresponding 17\(\beta\)-estradiol, \(p < 0.05\).
* Significantly different from placebo OVX animals, \(p < 0.05\).
* Significantly different from TCDD-treated intact and OVX TCDD 0.18 mg/pellet, \(p < 0.05\).
These effects were not observed in rats receiving placebo pellets. Following 9 weeks of release from subcutaneous pellets, rats receiving 17β-estradiol gained weight at a faster rate than intact rats. The alterations in body weight were likely the result of decreasing serum estradiol levels after 9 weeks of release and increasing serum estradiol levels after new pellets were implanted.

Liver weight. Increased liver weight is often associated with non-specific hepatotoxicity. No significant effects were observed on absolute liver weights regardless of ovariectomy or TCDD and 17β-estradiol treatment. Following 20 weeks of TCDD treatment, relative liver weight (g/liver/100g body weight) was increased in OVX and 17β-estradiol-supplemented OVX rats, but not intact rats as compared to corresponding corn-oil controls (Table 1). Following 30 weeks of treatment, the relative liver weights of TCDD-treated intact, OVX, and 17β-estradiol-supplemented OVX rats were all significantly higher than treatment-matched corn-oil controls.

Relative liver weights of OVX rats receiving placebo pellets were lower than intact rats or OVX rats receiving 17β-estradiol treatment. These observations more likely reflect decreasing effects of 17β-estradiol on body weight than increased liver weight leading to higher liver:body weight ratios as demonstrated by unaffected absolute liver weights in 17β-estradiol-treated rats.

Serum chemistry. Serum samples demonstrated a transient increase in hepatocellular toxicity in OVX rats co-treated with TCDD and 17β-estradiol compared to OVX rats receiving corn oil and 17β-estradiol-treated, as determined by the levels of alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) activity (Table 2). ALT and SDH activities in these rats were significantly higher following 20 but not 30 weeks of treatment. Hepatobiliary toxicity, as measured by total serum bile acids, was not significantly altered; however, serum 5′-nucleotidase (5ND) was significantly increased in OVX rats treated with TCDD as compared to those receiving corn oil, following both 20 and 30 weeks of treatment. 5ND activity was not significantly increased in TCDD-treated OVX rats exposed

### TABLE 2

<table>
<thead>
<tr>
<th>Dose group</th>
<th>17β-Estradiol (mg/pellet)</th>
<th>ALT (IU/L)</th>
<th>SDH (IU/L)</th>
<th>Bile acids (μM)</th>
<th>ALP (IU/L)</th>
<th>5ND (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>20 Weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact control</td>
<td>0</td>
<td>39.6 ± 16.6</td>
<td>19.0 ± 12.9</td>
<td>25.9 ± 21.3</td>
<td>23.4 ± 10.7</td>
<td>35.8 ± 12.0</td>
</tr>
<tr>
<td>Intact TCDD</td>
<td>0</td>
<td>49.8 ± 19.0</td>
<td>18.9 ± 5.5</td>
<td>38.5 ± 19.9</td>
<td>41.8 ± 22.2</td>
<td>41.1 ± 8.1</td>
</tr>
<tr>
<td>OVX control</td>
<td>0</td>
<td>33.7 ± 4.8</td>
<td>16.0 ± 3.8</td>
<td>21.6 ± 14.1</td>
<td>34.6 ± 9.6</td>
<td>30.1 ± 5.4</td>
</tr>
<tr>
<td>OVX TCDD</td>
<td>0</td>
<td>40.4 ± 7.1</td>
<td>20.4 ± 5.8</td>
<td>14.0 ± 6.1</td>
<td>49.8 ± 20.2</td>
<td>45.7 ± 10.1</td>
</tr>
<tr>
<td>OVX control</td>
<td>0.18</td>
<td>35.3 ± 6.0</td>
<td>12.9 ± 2.9</td>
<td>34.6 ± 13.0</td>
<td>12.0 ± 2.9</td>
<td>31.4 ± 10.2</td>
</tr>
<tr>
<td>OVX TCDD</td>
<td>0.18</td>
<td>58.4 ± 17.8</td>
<td>23.1 ± 6.5</td>
<td>40.0 ± 26.7</td>
<td>43.0 ± 27.3</td>
<td>37.1 ± 14.2</td>
</tr>
<tr>
<td><strong>30 Weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact control</td>
<td>0</td>
<td>46.4 ± 11.1</td>
<td>34.0 ± 21.0</td>
<td>59.7 ± 70.0</td>
<td>40.6 ± 20.6</td>
<td>56.9 ± 37.7</td>
</tr>
<tr>
<td>Intact TCDD</td>
<td>0</td>
<td>48.0 ± 9.6</td>
<td>37.9 ± 26.4</td>
<td>31.4 ± 11.1</td>
<td>38.5 ± 16.6</td>
<td>59.2 ± 40.7</td>
</tr>
<tr>
<td>OVX control</td>
<td>0</td>
<td>37.2 ± 7.3</td>
<td>27.3 ± 9.3</td>
<td>17.0 ± 7.4</td>
<td>33.0 ± 11.0</td>
<td>43.3 ± 8.7</td>
</tr>
<tr>
<td>OVX TCDD</td>
<td>0.18</td>
<td>65.5 ± 33.5</td>
<td>62.8 ± 50.5</td>
<td>37.3 ± 34.3</td>
<td>56.4 ± 25.9</td>
<td>93.9 ± 33.2</td>
</tr>
<tr>
<td>OVX control</td>
<td>0.18</td>
<td>42.0 ± 11.7</td>
<td>19.0 ± 5.2</td>
<td>28.3 ± 23.5</td>
<td>40.5 ± 25.3</td>
<td>34.8 ± 8.6</td>
</tr>
<tr>
<td>OVX TCDD</td>
<td>0.18</td>
<td>56.6 ± 29.3</td>
<td>39.4 ± 27.6</td>
<td>76.5 ± 113.2</td>
<td>45.8 ± 10.1</td>
<td>55.0 ± 23.9</td>
</tr>
</tbody>
</table>

*Note. Values are means ± standard deviation, 9 animals per group. OVX, ovariectomized; ALT, alanine aminotransferase; SDH, sorbitol dehydrogenase; ALP, alkaline phosphatase; 5ND, 5′-nucleotidase.

*a Significantly different from corresponding estrogen without TCDD control, p < 0.05.

*b Significantly different from OVX placebo control, p < 0.05.
to 17 β-estradiol or TCDD-treated intact rats at either time point. Alkaline phosphatase (ALP) activity, another measure of hepatobiliary toxicity, was significantly higher in TCDD-treated intact rats and TCDD-treated 17 β-estradiol-supplemented OVX rats as compared to corresponding corn-oil controls following 20 weeks of treatment. There was no significant increase in TCDD-treated OVX rats receiving placebo pellets, following 20 weeks of treatment. In OVX rats receiving corn oil, ALP activity was significantly lower in rats receiving 17 β-estradiol than in rats receiving placebo pellets. No significant treatment-related alterations of ALP activity were observed following 30 weeks of treatment, indicating that the effects of treatment on ALP activity were transient.

Toxicity and histopathology. Three incidences of mortality occurred in the study. Mortality was observed in one animal following diethylnitrosamine (DEN) initiation prior to TCDD exposure. Isolated incidences of mortality also occurred in two OVX rats receiving 0.18 mg 17 β-estradiol/pellet, following 15 weeks of TCDD treatment and 26 weeks of corn oil, respectively. Animal deaths were due to unknown causes. No treatment-related mortality was observed.

Control and TCDD-exposed livers contained variable amounts of intracellular glycogen. In control animals, the glycogen appeared widespread while in TCDD-exposed animals there was an apparent loss of glycogen in the periportal areas. Exposure to TCDD also seemed to result in increased chronic inflammation, the centrilobular deposition of a hemosiderin-like pigment, and increased numbers of degenerated hepatocytes. In general, exposure to TCDD resulted in the presence or increased incidence of a number of lesions, which included hepatocellular cytoplasmic vacuolization, hepatocellular cytomegaly, and hepatocellular syncytial alteration (Table 3). These lesions were present following 20 weeks of treatment, but the severity was comparatively increased following 30 weeks of treatment.

Hepatocellular cytoplasmic vacuolization was characterized by the presence of variable-sized, clear, cytoplasmic droplets within centrilobular hepatocytes. The severity of cytoplasmic vacuolization was increased in OVX TCDD placebo groups following both 20 and 30 weeks of treatment. In addition, OVX control animals also had cytoplasmic vacuolization. Hepatocellular cytomegaly was characterized by an overall cytoplasmic and nuclear size increase adjacent to centrilobular regions. In general, the severity of cytomegaly was reported to be minimal. The presence of hepatocellular syncytial alteration was a hallmark of TCDD exposure since this change was not observed in any of the controls. Syncytial alteration was characterized by the presence of large, often irregularly shaped hepatocytes that contained multiple nuclei. The cytoplasm of these altered cells appeared densely eosinophilic. Although their distribution appeared random, many were apparent near periportal regions. Estrogen supplementation did not seem to have any effect on the incidence or severity of this change.

Control and TCDD-exposed livers contained variable amounts of intracellular glycogen. In control animals, the glycogen appeared widespread while in TCDD-exposed animals there was an apparent loss of glycogen in the periportal areas. Exposure to TCDD also seemed to result in increased chronic inflammation, the centrilobular deposition of a hemosiderin-like pigment, and increased numbers of degenerated hepatocytes. In general, exposure to TCDD resulted in the presence or increased incidence of a number of lesions, which included hepatocellular cytoplasmic vacuolization, hepatocellular cytomegaly, and hepatocellular syncytial alteration (Table 3). These lesions were present following 20 weeks of treatment, but the severity was comparatively increased following 30 weeks of treatment.

Hepatocellular cytoplasmic vacuolization was characterized by the presence of variable-sized, clear, cytoplasmic droplets within centrilobular hepatocytes. The severity of cytoplasmic vacuolization was increased in OVX TCDD placebo groups following both 20 and 30 weeks of treatment. In addition, OVX control animals also had cytoplasmic vacuolization. Hepatocellular cytomegaly was characterized by an overall cytoplasmic and nuclear size increase adjacent to centrilobular regions. In general, the severity of cytomegaly was reported to be minimal. The presence of hepatocellular syncytial alteration was a hallmark of TCDD exposure since this change was not observed in any of the controls. Syncytial alteration was characterized by the presence of large, often irregularly shaped hepatocytes that contained multiple nuclei. The cytoplasm of these altered cells appeared densely eosinophilic. Although their distribution appeared random, many were apparent near periportal regions. Estrogen supplementation did not seem to have any effect on the incidence or severity of this change.

### DISCUSSION

The toxicity of co-treatment with TCDD and 17 β-estradiol, and the assessment of the efficacy of 90-day subcutaneous 17 β-estradiol constant-release pellets, were determined. These results demonstrate that although the release of 17 β-estradiol from 90-day subcutaneous pellets was not constant in DEN-initiated female Sprague-Dawley rats, the 17 β-estradiol pellets were effective in inducing estrogen-sensitive responses. Furthermore, biological responses to 17 β-estradiol supplementation in OVX rats such as alterations in body weight and uterine weight were similar to intact rats. Supplemental 17 β-estradiol did not exhibit overt toxicity when co-administered with TCDD. Chronic exposure to TCDD resulted in significantly higher hepatic concentrations of TCDD in OVX rats than in intact rats after 30 weeks of treatment. These results are consistent with previously observed hepatic concentrations of TCDD in intact and OVX female Sprague-Dawley rats (Lucier et al., 1991). Similar hepatic TCDD concentrations in 17 β-estradiol-supplemented OVX rats and intact rats suggest that 17 β-estradiol exposure affects hepatic concentrations of TCDD. Hepatic TCDD concentrations in intact rats receiving only corn oil were 2-fold higher than OVX rats regardless of 17 β-estradiol treatment. A combination of ovariectomy-related effects such as increased body weight gain, increased body fat mass (Bagi et al., 1995), and the possible redistribution of fat (Clark and Tarttelin, 1982) may explain these differences. Since TCDD is highly lipophilic, the pharmacokinetics and distribution of low levels of TCDD ingested in the feed (Van den Heuvel et al., 1994) are likely altered in OVX rats. Unlike in TCDD-exposed rats, the effects of ovariectomy on TCDD concentrations in control rats are not mitigated by 17 β-estradiol exposure.

Higher body weights in OVX rats compared to intact rats and decreased body weights in TCDD-treated rats are consis-
tent with previous findings of the effects of TCDD and ovariectomy on body weight gain in female Sprague-Dawley rats (Harris et al., 1973; Kociba et al., 1976; Lucier et al., 1991). However, in contrast to previous findings (Lucier et al., 1991), significantly decreased body weights were observed in TCDD-treated OVX rats; the reason for this is not clear. However, it is unlikely to be due to the dosing regimen. The present study used a lower weekly dose of 700 ng/kg regimen of approximately a 100-ng/kg/day dose compared to a biweekly dose of 1400 ng/kg/day used previously (Lucier et al., 1991). Furthermore, mean concentrations of TCDD in the livers of OVX rats in the present study (16,769 ppt) were lower than previously observed in OVX rats (34,000 ppt) (Lucier et al., 1991). The body weights of 17 β-estradiol-supplemented OVX rats were within a similar range to that of intact rats.

TCDD exposure resulted in a significant decrease of serum estradiol concentration in intact rats following 20 weeks of treatment. However, following 30 weeks of TCDD treatment, serum estradiol concentrations in intact rats were slightly higher, but not significantly different from controls. No alterations of serum estradiol levels were observed in OVX rats or 17 β-estradiol-supplemented OVX rats. The lower serum estradiol concentrations may be a result of metabolism by TCDD-induced cytochromes P450 A1A and 1A2. However, since serum estradiol levels are highly variable and dependent upon progression through the estrous cycle, these results may reflect a greater percentage of control rats in the proestrous phase of the estrous cycle. These results are consistent with observations in CD-1 mice (DeVito et al., 1992) and pregnant Holtzman rats (Shiverick and Muther, 1983), which demonstrate no significant alteration of serum estradiol following exposure to TCDD.

Several clinical observations confirm the efficacy of supplemental subcutaneous 17 β-estradiol pellets on the induction of estrogenic responses. Uterine wet weights in OVX rats receiving all doses of 17 β-estradiol were within the range of observed uterine wet weights for intact rats. Terminal serum 17 β-estradiol levels (37–68 pg/ml) were within the range of previously observed physiological levels of 1.2–88 pg/ml (Overpeck et al., 1978). However, 17 β-estradiol pellet implants resulted in initial supra-physiological serum concentrations. Although terminal serum estradiol concentrations were within the expected range, the daily serum estradiol concentrations decreased from >300 pg/ml 14 days after implantation to target concentrations at termination of the study. Previous studies using subcutaneous pellets have only reported terminal concentrations of estradiol, not interim concentrations. The fluctuation in serum estradiol concentration was reflected in the change of body weights (Fig. 2). Decreases in body weight were observed in periods of high serum-estradiol concentrations. The increase of body weight in the period corresponding to decreased serum estradiol suggests that the pellets released 17 β-estradiol at a slower rate towards the end of the ninety days. The decrease in serum 17 β-estradiol levels may result from discontinuous release of 17 β-estradiol from the pellet. Discontinuous serum levels of 17 β-estradiol may also be attributed to age-dependent estrogen metabolism as well as a decreased rate of dose/kg as rats gained weight over the course of pellet release. The discontinuous serum concentrations of 17 β-estradiol are likely a result of a combination of these alterations over the 90-day period of release. These data demonstrate the necessity to monitor serum estradiol levels throughout the 90-day release period and not solely the terminal concentrations.

Clinical chemistry and histopathology results indicate no excess hepatotoxicity is associated with 17 β-estradiol treatment in TCDD-exposed OVX female Sprague-Dawley rats. Exposure to TCDD either resulted in the presence or increased incidence of a number of toxicologic-induced histopathological changes, which provide evidence for a spectrum of TCDD-induced hepatotoxicity. However, for most lesions, there did not appear to be any significant differences between OVX TCDD-treated rats receiving 17 β-estradiol supplementation and those not receiving 17 β-estradiol. While the present study did not reveal alterations in the toxicity of TCDD with combined treatment, other studies have demonstrated the potential lethality of TCDD by tamoxifen in CD-1 mice (Umbreit et al., 1989; MacKenzie et al., 1992).

The current study demonstrates that the supplemental estrogen model provides a useful tool in elucidating the role of 17 β-estradiol in TCDD-induced hepatocarcinogenesis. TCDD concentrations were equivalent in livers of sham operated and OVX TCDD-treated rats as well as OVX rats co-treated with TCDD and 17 β-estradiol. Although serum 17 β-estradiol concentrations were not constant and resulted in initially supra-physiological levels, uterine weights and total body weights in OVX rats receiving supplemental 17 β-estradiol were comparable to sham-operated female rats. There was no additional hepatotoxicity associated with co-treatment of 17 β-estradiol and TCDD as compared to treatment with TCDD alone, as evidenced by serum chemistry and histopathology. Ongoing analyses are investigating if 17 β-estradiol enhances hepatocyte proliferation and the formation and development of altered hepatocellular foci in TCDD-treated female rats. Additionally, the added capacity for the formation of reactive oxygen species from redox cycling of catechol estrogens and the subsequent enhancement of 8-hydroxydeoxyguanosine oxidative DNA adducts in TCDD-treated female Sprague-Dawley rats concurrently exposed to 17 β-estradiol are now being evaluated. These data will aid in gaining insight into the relationship between early molecular events such as the induction of cytochrome P450 and subsequent pathological responses.

ACKNOWLEDGMENTS

The authors gratefully thank Louise Harris, Larry Judd, James Clark, Bill Ross, Greg Travlos, Ralph Wilson, Scott Masten, Amy Kim, Diane Spencer, Ben Willems, Page Myers, Jean Grassman, and Heather Vahdat for technical assistance.
REFERENCES


