Adult 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) Exposure and Effects on Male Reproductive Organs in Three Differentially TCDD-Susceptible Rat Lines

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The contribution of genetic factors to adult male reproductive system toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was analyzed in three rat lines differentially resistant to TCDD acute lethality: line A, B, and C rats (selectively bred from TCDD-resistant Han/Wistar [Kuopio; H/W] and TCDD-sensitive Long-Evans [Turku/AB; L-E] rats). The resistance is linked to a mutated H/W-type aryl hydrocarbon receptor allele in line A and to an H/W-type unknown ‘’B’’ allele in line B. Line C rats do not have resistance alleles. Mature male line A, B and C rats were given single oral doses up to 1000, 300, and 30 µg/kg TCDD, respectively. The dose-responses of TCDD effects on male reproductive organ weights, sperm numbers, and serum testosterone concentrations were analyzed 17 days after exposure. Serum testosterone concentrations were decreased by the highest doses of TCDD, and there were no major sensitivity differences among the rat lines. Correspondingly, the decrease in relative weight of ventral prostate and seminal vesicles was seen only after a dose of >100 µg/kg TCDD. Thus the effect was observed only in resistant lines A and B. The relative weights of testes and epididymides were not affected. Significant decrease in spermatogenesis was observed in each rat line, but the amount of decrease was reduced by resistance alleles. The highest TCDD dose decreased the daily sperm production by 37, 38, and 60% in line A, B, and C rats, respectively. Therefore, the resistance alleles appear to selectively modify the TCDD effects on the adult male reproductive system. The fact that the influence of resistance alleles on spermatogenesis is different from that on androgenic status indicates that the effect of TCDD on sperm numbers is not fully related to decreased serum testosterone.

Key Words: dioxin; TCDD; strain differences; adult exposure; male reproductive toxicity.

Exposure of experimental animals to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes a set of diverse toxicological and biological effects in most vertebrates studied thus far. However, the sensitivity to TCDD effects and the pattern of effects seen may vary among animal species and even within the same species, depending on the strain used. Male reproductive toxicity is one of the most sensitive and extensively studied toxic endpoints of TCDD that is considered to belong to a group of environmental endocrine disrupters. Although the developing male reproductive system is known to be highly sensitive to TCDD, decreased testsis and accessory sex organ weights, dissolution of germinal epithelium, degeneration of germ cells, decreased spermatogenesis, and reduced fertility have also been reported in TCDD-treated adult animals (Chahoud et al., 1992; Peterson et al., 1993).

In our laboratory we have used a rat model based on an exceptionally wide (>1000-fold) sensitivity difference between the sensitive Long-Evans (Turku/AB) (L-E) rats and the resistant Han/Wistar (Kuopio) (H/W) rats in terms of acute lethality of TCDD. A point mutation in the H/W-type aryl hydrocarbon receptor (Ahr) allele (Ahrhw; hw denoting allele originating from H/W rats) results in an abnormal C-terminus transactivation domain and a smaller AHR protein compared to that of normal rat strains (Ahrwt; wt denoting wild-type allele) (Pohjanvirta et al., 1998; 1999). The exceptional resistance of H/W rats to TCDD acute lethality is mostly associated with this abnormal AHR and, to a lesser extent, with an unknown allele “B” (Tuomisto et al., 1999).

Recently, these two H/W-type “TCDD resistance alleles” (Pohjanvirta 1990; Tuomisto et al., 1999), were segregated into two new rat lines and they were designated lines A and B (Tuomisto et al., 1999). Line A has the resistant Ahr allele (Ahrhw) and line B has the resistant B allele present in H/W rat (Bhw). Line C has only wild-type alleles Ahrwt/Bwt. Lines A, B, and C exhibit highly different LD50 values for TCDD: >10,000, 830, and 40 µg/kg in male rats, respectively.

Our recent dose-response analysis showed that the typical endpoints of dioxin short-term toxicity can be classified on the basis of efficacy modification by the resistance alleles Ahrhw and Bhw (Simanainen et al., 2002; 2003). The type I endpoints
(increased EROD activity, decreased thymus weight, and tooth defect) are independent of genotype variation, and the rat lines respond similarly to TCDD exposure. For type II endpoints (e.g., weight loss, liver toxicity, and increased serum bilirubin) the magnitude of effect is suppressed by at least 50% owing to the resistance alleles, and in general the sensitivity to these endpoints among the rat lines follow the same ranking order as that of LD50 values.

The Ahrbw allele proved to be the most important resistance factor. The C-terminus of AHR is required for the formation of functional AHR conformation, which increases promoter accessibility and facilitates promoter occupancy by different transcription factors (Ko et al., 1996; Kronenberg et al., 2000). Because the promoter sequences and the transcription factor machinery vary among genes, it could be hypothesized that the mutation in the Ahrbw selectively affects the formation of functional AHR conformation as well as communication between enhancer and promoter, leading to altered expression of different genes.

On the other hand, the long-term effects may not follow the classification criteria developed for short-term effects (cf. Viluksela et al., 2000). After a 20-week exposure to TCDD, the H/W and L-E rats showed little difference in efficacy of TCDD but about a 100-fold difference in potency of TCDD for increased plasma aspartate aminotransferase activity (Viluksela et al., 2000), which is a typical type II endpoint based on short-term experiments (Simanainen et al., 2002; 2003). This implies that the primary difference is in efficacy, and it is observed first, whereas toxicity or tolerance manifested after several weeks may modify the effect, leading to secondary differences in potency, which is also seen in acute lethality.

In the previous study with line A, B, and C rats, we demonstrated that the male reproductive organ defects after in utero and lactational TCDD exposure were not profoundly affected by the resistance alleles. However, the decrease in daily sperm production and cauda epididymal sperm number was reduced by the resistance alleles (Simanainen et al., 2004). The maternal dose of 1 µg/kg TCDD on gestation day 15 reduced daily sperm production by 9.3, 25, and 36%, and cauda epididymal sperm reserves by 18, 42, and 49% in line A, B, and C rats when measured on PND 70, respectively.

In the present study we analyzed the effects of adult TCDD exposure on the structure and function of male reproductive organs. The objective was to distinguish the roles of the resistance alleles Ahrbw and Ahbh in adult male reproductive effects following exposure to TCDD.

**MATERIALS AND METHODS**

**Chemicals.** TCDD was purchased from the UFA-Oil Institute (Ufa, Russia) and was >99% pure, as confirmed by gas chromatography–mass spectrometry. The TCDD was weighed and dissolved in diethyl ether. Adjusted volume of diethyl ether was mixed with corn oil and ether was let to evaporate. Dosing solutions were mixed in a magnetic stirrer and sonicated for 20 min before dosing.

Methanol and diethyl ether were of analytical grade and purchased from Merck (Darmstadt, Germany) and from BDH Laboratory Supplies (Poole, England), respectively.

**Animals and animal husbandry.** Adult male line A, B, and C rats were obtained from the breeding colony of the National Public Health Institute (Kuopio, Finland). The crossing protocol to develop these lines has been described earlier (Tuomisto et al., 1999).

The rats were housed in stainless steel wire-bottom cages, 1–3 rats per cage, and given commercial rat chow (R36; Lactamin, Stockholm, Sweden) and tap water ad libitum. The ambient temperature in the animal room was 21 ± 1°C, and the relative humidity was 55 ± 10%. The rats were kept under a photoperiodic cycle of 12 h light per 12 h dark in an air-conditioned animal room.

**Experimental design.** Line A, B, and C rats were randomly divided into experimental groups of 5–6 (5.9 ± 0.3; mean ± SD) animals. Male rats were 12–16 weeks old at the beginning of the experiment. Line A rats weighed 280 ± 5.6 g (mean ± SD), line B rats weighed 336 ± 22 g, and line C rats weighed 288 ± 7.3 g. The dosing groups and the number of animals per dosing group are presented in Table 1. To establish dose-responses, the rats were given a single oral dose of TCDD in corn oil by oral gavage (4 ml/kg) at several dose-levels. Because of different sensitivities of the rat lines, the TCDD doses were between 0.1 and 1000, 0.1 and 300, and 0.1 and 30 µg/kg body weight in line A, B, and C rats, respectively. Control animals were dosed similarly with corn oil vehicle. The lower maximal doses in line B and line C rats compared to the doses in line A rats were chosen to keep the doses below the respective LD50 values of each rat line.

Because the length of the seminiferous epithelial cycle in the rat is ~13 days, the rats were euthanized at day 17 postexposure. All animals were euthanized between 9 a.m. and noon. Rats were anesthetized with CO2, and blood collected with heart puncture. Serum was separated and stored at −80°C until analyses. Testes, right epididymis (cauda epididymis), ventral prostate, and seminal vesicles (emptied) were quickly removed, trimmed, and weighed. The organ weights were reported as both absolute and relative weights (organ weight/body

**TABLE 1**

<table>
<thead>
<tr>
<th>Line</th>
<th>Dose (µg/kg TCDD)</th>
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weight). Right testis, half of the ventral prostate, and cauda epididymis were quickly frozen in liquid nitrogen and stored at −80°C.

**Serum testosterone concentration.** Testosterone was measured from diethyl ether extracted sera by time-resolved fluorimunnoassay, DELFIA (Perkin Elmer Life and Analytical Sciences, Wallac Oy, Turku, Finland). After extraction, samples were reconstituted to 100 μl of Dilution II buffer (Perkin Elmer Life and Analytical Sciences) from which 25 μl per well was taken for analysis. For enhancing the sensitivity of the assay, commercial tracer and antisera were additionally diluted 5:8 giving the sensitivity of >0.04 ng/ml when the limit of detection was defined as the value 2 SD above the mean of the zero standard measurement values.

**Testis morphometry.** For fixation, the left testis was immersed in Bouin’s solution. After 8 h the tissues were partially sliced and further fixed in fresh Bouin’s solution for 24 h before washing in 70% ethanol. Testes were sectioned at 5-μm thickness and stained with periodic acid-Schiff–hematoxylin (PAS-H). For all control and the highest TCDD-dosed animals, one section from one testis was examined. One investigator (U. S.) without knowledge of dose examined all sections. The diameter of 10-stage VII/VIII seminiferous tubules per section was analyzed with computer-assisted light microscopy by using Stereo-Investigator and NeuroExplorer (MicroBrightField, VT) software. The numbers of Sertoli cells, spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, and round spermatids were quantified in five round cross-sections of seminiferous tubules at stages VII-VIII of the epithelial cycle by using 60x magnification in Olympus (Japan) light microscope. Results are presented as cell numbers per cross-section.

**Daily sperm production and epididymal sperm head count.** For sperm counts, the frozen testes and right cauda epididymis were homogenized separately for 2 min using IKA Ultra Turrax (model T25 basic) in 50 ml (testes) or 20 ml (cauda epididymis) 0.9 % saline, containing 0.05% Triton X-100 and 0.01% thimerosal. Homogenates were diluted to approximately 1 × 10⁶ sperm/ml, and counts from four hemocytometer chambers were counted and averaged.

**Statistical analysis.** Statistical analysis was performed by using the one-way analysis of variance (ANOVA). If this test showed a significant difference, the least significant difference test was used as a post hoc test. In case of nonhomogenous variances (according to Levene’s test, p < 0.01), the nonparametric Kruskal-Wallis ANOVA was used followed by the Mann-Whitney U-test. P values <0.05 were considered significant. The data from testis morphology were analyzed by using an independent samples t-test.

**RESULTS**

Body weight was significantly decreased at the highest doses in all lines (Fig. 1). The decrease was maximally 6.6, 17, and 14 % compared to control line A, B, and C rats, respectively.

**Androgenic Status**

Serum testosterone levels were decreased by TCDD exposure (Fig. 2). The maximal decreases were 91, 43, and 60% in line A, B and C rats, respectively. The relative ventral prostate weight was decreased only at the highest TCDD doses in line A and B rats (Fig. 3). The ventral prostates in TCDD-exposed males were maximally 54, 64, and 25% smaller than those in control males of line A, B, and C rats, respectively. Similarly, the relative seminal vesicle weight was decreased only at the highest TCDD doses (Fig. 3).

**Effects on Testis and Epididymis**

Testis weight was not affected by TCDD. Data are shown only for control and highest TCDD dose group in Table 2. The highest dose of TCDD significantly decreased the number of Sertoli cells, round spermatids, and preleptotene spermatocytes per tubule only in line B rats. The number of pachytene spermatocytes was significantly decreased at the highest TCDD dose in all rat lines (Fig. 4). The number of spermatogonia per tubule was not affected. The relative weight of epididymis was not affected by TCDD (Fig 5). Relative cauda epididymis weight was significantly decreased only in line A rats dosed with 1000 μg/kg TCDD (Fig. 5).
Sperm Parameters

The daily sperm production in the testes was dose-dependently decreased in each line. At the highest dose, the daily sperm production was decreased by 37, 38, and 60% in line A, B, and C rats, respectively (Fig. 6). The two highest TCDD doses significantly decreased epididymal sperm reserves in each line (Fig. 6). The decrease was maximally 34, 55, and 35%, in line A, B, and C rats, respectively.

DISCUSSION

In recent years, the studies on the TCDD-induced male reproductive toxicity has been focused on effects of perinatal TCDD exposure. The adult male reproductive system is also affected by TCDD exposure, although during development the reproductive system is about 100–fold more susceptible (Peterson et al., 1993). As seen in general TCDD toxicity, genetic diversity among animal species as well as within one species may cause differences in sensitivity and toxic outcomes. In a previous study we demonstrated that the sensitivity to TCDD-induced decrease in sperm numbers after developmental exposure was reduced by resistance alleles (Simanainen et al., 2004). Similarly in the present study, the resistance alleles appear to modify the TCDD effect on sperm numbers, but not on male reproductive organs.

Moore and coworkers (1985) reported an ED50 value of 15 μg/kg TCDD for the decrease in plasma androgen levels in sexually mature Sprague-Dawley rats. The effect was measured 7 days postexposure and persisted for at least 12 days postexposure. In the present study, the serum testosterone was measured 17 days postexposure and the concentrations were reduced by resistance alleles (Simanainen et al., 2004). Similarly in the present study, the resistance alleles appear to modify the TCDD effect on sperm numbers, but not on male reproductive organs.
The testosterone concentration was only 10% of control levels. Such a great decrease in testosterone levels is biologically relevant and should already result in decreased weights of androgen-dependent organs like seminal vesicles and prostate.

The relative ventral prostate and seminal vesicle weights were most markedly decreased at doses of $4 \times 10^3$ mg/kg TCDD, reflecting the decrease in androgen levels. The weight of accessory sex organs is primarily dependent on the dihydrotestosterone (DHT) that is converted from testosterone by 5α-reductase. Moore and coworkers (1985) showed that in Sprague-Dawley rats also the serum DHT levels were decreased and that the decrease appeared to be related to the decrease in serum testosterone rather than to decreased activity of 5α-reductase. The results indicate that the resistance alleles do not reduce the effects of TCDD to androgenic endpoints at adulthood. The TCDD potency appeared to be similar among the rat lines and the magnitude of decrease in organ weights was even larger in line A and B rats than in C rats.

Rat studies with postpubertal TCDD exposure reported unchanged testis weights or even an increase in the relative testis weights resulting from decreased body weight at higher doses (Chahoud et al., 1992; Johnson et al., 1992; Moore et al., 1985; Rune et al., 1991; Tofilon and Piper, 1982). Similarly, there was no effect on testis weights in line A, B, and C rats in the present study. In contrast, previous studies reported significant decreases in testis weight after in utero and lactational TCDD exposure (reviewed by Roman and Peterson, 1998), or when the rats were exposed at puberty (el-Sabeawy et al., 1998; Latchoumycandane et al., 2002). Therefore, the critical period for TCDD effects on testis weight appear to be before puberty.

In spite of unaffected testis weight, disturbed morphology has been reported to be the most conspicuous effect after adult TCDD exposure (Chahoud et al., 1992; Johnson et al., 1992; Rune et al., 1991). In the present study, no major histopathological lesions were observed. Only a small decrease in the number of Sertoli cells and germ cells was observed at the highest TCDD doses, but it was statistically significant only in line B rats. Thus, the declined sperm numbers in epididymis suggest both slight testicular damage and increased post testicular demise of the sperm cells.

In line with previous studies (Moore et al., 1985; Rune et al., 1991), spermatogenesis was affected even at doses lower than those causing decreased serum androgen levels and decreased size of accessory sex organs. TCDD most significantly decreased the daily sperm production in line A rats, already with a dose of 1 μg/kg TCDD (Fig. 6). However, the magnitude of decrease in daily sperm production was reduced by the resistance alleles and, was almost two-fold in line C rats compared to line A and B rats. The decrease of cauda epididymal sperm numbers was smaller in line A rats at 10 μg/kg than in the other rat lines, but the overall efficacy did not show differences among the rat lines.

Taken together, there seems to be sensitivity differences between line A and C rats, and this suggests that the TCDD effects on sperm parameters are modified by the mutated H/W-type AHR. The definite classification of effects into types I and II was not possible by the classification criteria set for short-term toxic endpoints of TCDD exposure (Simanainen et al., 2002; 2003). However, the resistance towards acute effects, including lethality, in the resistant lines makes it possible to study complete dose-responses of adverse effects. This may enable an improved characterization of toxicity and its mechanisms, including how TCDD decreased daily sperm production.

![FIG. 4. Effect of TCDD on number of Sertoli cells and spermatogenic cells per seminiferous tubule cross-section in line A, B, and C rats (group mean ± SE; 5–6 animals per treatment group). * Indicates statistically significant difference compared to respective control analyzed by student $t$-test.](https://academic.oup.com/toxsci/article-abstract/81/2/401/1656100)
In conclusion, the data suggest that androgenic status is unaffected by the HW-type resistance alleles, whereas sperm parameters are affected especially by the H/W-type AHR. Thus, the role of resistance alleles in adult TCDD exposure appears to be similar to that in perinatal exposure. In addition, the present data support the conclusion that the effects on adult accessory sex organ weights are related to decreases in testosterone concentrations and/or androgen responsiveness (Bookstaff et al., 1990; Moore et al., 1985). However, the results suggest that the effects on sperm parameters are not completely caused by decreased serum testosterone levels, as the resistance alleles differentially modify the effect of TCDD on serum testosterone and spermatogenesis. Furthermore, the critical period for TCDD effects on testis weight appears to occur before puberty, and

**FIG. 5.** Effects of TCDD on absolute (g) and relative (mg/g body weight) weight of epididymis and cauda epididymis in line A, B, and C rats, 17 days postexposure (group mean ± SE; 5–6 animals per treatment group). For group means statistically significant ($p < 0.05$) differences versus corresponding controls are depicted with solid symbols. Statistical analysis was performed by one-way ANOVA.

**FIG. 6.** Effects of TCDD on daily sperm production ($10^6$ sperms/testis) and cauda epididymal (CE) sperm reserve ($10^6$ sperms CE) in line A, B, and C rats, 17 days postexposure (group mean ± SE; 5–6 animals per treatment group). For group means statistically significant ($p < 0.05$) differences versus corresponding controls are depicted with solid symbols. Statistical analysis was performed by one-way ANOVA.
there seems to be no effect on testis weight, if the exposure occurs during the adulthood.

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