Maternal Exposure of Low Dose of TCDD Modulates the Expression of Estrogen Receptor Subunits of Male Gonads in Offspring

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ABSTRACT. We have analyzed the effects of low-dose transplacental and lactational exposure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on gene expression relating to the dioxin and sexual hormone cascade, and demonstrated the effects on testicular growth and sexual maturation in male offspring rats. TCDD (10 *n*g/kg) was administered to dams on Days 7 and 14 of gestation, and on Days 0, 7 and 14 after delivery. Gene expression of cytochrome P450 family 1 subfamily A polypeptide 1 (CYP1A1) in the liver of 17-day-old rats was significantly increased compared with controls. Furthermore, expression of estrogen receptors (ER)α and ERβ was significantly increased at 17 and 42 days old, respectively in the testis of TCDD-administered rats compared with controls. Although testicular weight and the seminiferous tubule diameter were increased in 17-day-old rats, there was no difference in the number of germ cells between TCDD-treated and control animals. The expressions of androgen receptor and inhibin subunit genes were not significantly changed. These findings suggest that low-dose exposure of TCDD leads to unusual development of the testis by perturbation of steroid hormone homeostasis.
KEY WORDS: CYP1A1, estrogen receptor, gene expression, spermatogenesis, TCDD.

TCDD is the most potent congener of the polychlorinated dioxins and is regarded as one of the important endocrine disruptors. Toxic effects of TCDD are mediated by the aryl hydrocarbon receptor (AhR) that binds TCDD with high affinity and heterodimerizes with the AhR nuclear translocator (ARNT) [7, 15, 24, 26, 34].

Maternal exposure to high dose of TCDD causes many irreversible abnormalities in various organs of fetus and offspring. Many researchers have reported that transplacental and lactational exposure to TCDD results in adverse changes to the male reproductive system [1, 2, 6, 11, 16, 17, 28, 32]. Many of these studies, however, were conducted using relatively high doses of TCDD. The effects of low-dose TCDD exposure have been reported by Peterson *et al.* [25], Ohsako *et al.* [21], and others [6, 8, 13, 31]. The expression of AhR in rats was increased by low-dose TCDD administration [8], and apoptosis of tubular cells was induced following depression by follicle stimulating hormone (FSH) [30]. Significant reductions were detected in testicular weight, daily sperm production and sperm numbers after *in utero* exposure of male rats to a single low dose of TCDD [25]. Using the same study protocol, the amounts of TCDD transferred to offspring from dams were found to be proportional to the injected amounts of 12.5 to 800 *n*g/kg, and furthermore, androgen receptor (AR) mRNA levels in the ventral prostate were decreased by the lowest dose of TCDD [21]. Faqi *et al.* [6] reported that treatment of dams with low doses of TCDD throughout pregnancy and lacta-

tion decreased the number of sperm and increased the number of abnormal sperm in offspring at puberty and adulthood. Conversely, several study groups reported that *in utero* exposure of high dose of TCDD did not reduce daily sperm production of rat offspring [35], and that testicular weight of offspring was not changed by subchronic *in utero* exposure [4]. Thus, the effects of maternal exposure of TCDD on testicular function of offspring remain controversial.

Since environmental exposure to TCDD is usually of a repeated persistent low-dose nature, the aim of this study is to examine effects on the offspring to maternal rats receiving comparable exposure. In the present study, we injected low-dose TCDD into pregnant rats, once per week, from fetal sex differentiation until they were weaned, and observed changes in gene expression in the testis and liver of male offspring.

MATERIALS AND METHODS

Animals: Seven pregnant Slc. Wistar rats were obtained on Day 3 of gestation (the day of sperm detection in vaginal smears was considered as Day 0). Dams were housed individually in an air-conditioned animal room at a temperature of 22°C, relative humidity of 55 ± 10%, and a 12-hr light/12-hr dark cycle. Animals received food and water *ad libitum*. Animal experiments using TCDD in this study were approved by the University of Yamanashi Animal Care and Use Committee.

TCDD administration to animals and morphology of the testis: TCDD (AccuStandard, Germany) was preserved in toluene solution (1 mg/m*l*). The stock solution was finally

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made up to a concentration of 100 *n*g TCDD/m*l* diluted in corn oil. Four of the dams were treated with TCDD and 3 controls were treated with vehicle of the same volume. TCDD was administered subcutaneously to dams at a dose of 10 *n*g/kg body weight (0.02–0.03 m*l*/injection) once per week (for a total of 5 times), on Days 7 and 14 of gestation and on Days 0, 7 and 14 after delivery. Offspring were weaned 21 days after birth, thereby terminating TCDD exposure from lactation. The 4 dams treated with TCDD delivered 42 offspring (17 males and 25 females) and the 3 controls delivered 34 offspring (16 males and 18 females). Randomly selected offspring were used for the following experiment. Nine male offspring from each of TCDD-treated group and control group were sacrificed at 17 days after birth, and others at 42 days after birth. External anomalies were examined, including anogenital distance and macroscopic organ anomalies. Body and right testicular weights were measured. The testis was examined histologically according to the procedure previously reported [23, 29].

Slices of testis were hematoxylin-eosin (H-E) stained. The numbers of germ cells per seminiferous tubule, Sertoli cells and Leydig cells were counted of stage XI at 42 days old, and the diameter of round seminiferous tubules (15 tubules per testis) at 17 and 42 days old were measured under a microscope.

Analysis of gene expression: Total RNA was isolated from the testis with Qiagen's RNeasy Mini Kit (Qiagen K.K. Japan). For amplification of the target genes, reverse-transcription (RT) and polymerase chain reaction (PCR) were run in two separate steps. Briefly, equal amounts of total RNA (1 μg) were used for RT in a final volume of 20 μl, containing avian myeloblastosis virus (AMV)-RT buffer, 5 U AMV-reverse transcriptase (RTase) (Takara

Co., Ltd., Japan), 20 U ribonuclease inhibitor, 1 mM dNTP mixture, and 50 pmol random hexamer primers. Reaction mixtures were incubated at 30°C for 10 min, followed by 42°C for 30 min, and finally heated at 97°C for 5 min, to inactivate the RTase. The cDNA products were stored at –20°C until use. Sequences and annealing temperatures of the primer sets are given in Table 1. The sequences of these primers were designed according to the DNA database. Accession numbers of these genes are as follows; CYP1A1: NM_012540, AhR: NM_013149, ARNT: NM_012780, inhibinα: NM_012590, inhibinβ-A: NM_017128, inhibinβ-B: XM_001053684, ERα: NM_012689, ERβ: NM_012754, AR: NM_012502, glyceraldehyde 3-phosphate dehydrogenase (GAPDH): NM_017008. The cDNA in accordance with 100 *n*g RNA was amplified in PCR buffer in the presence of 1.5 mM MgCl$_2$, 2.5 U Taq DNA polymerase, 200 nM dNTP mixture, and 10 nM each primer. PCR cycle conditions were as follows; the first denaturing cycle at 97°C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 97°C for 0.5 min, annealing of the primers to the cDNA at appropriate temperature (Table 1) for 1 min, and fragment extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR conditions were applied in this exponential range for semi-quantitative analysis. Different cycles were tested for all genes to define the exponential range, and appropriate cycles were chosen for further analysis (data not shown).

PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide staining. Stained gels were placed on a UV transilluminator and recorded with a CCD camera. Images were examined using gel and blot analysis software (ImageGauge software, Fuji Film Co., Ltd., Japan). Values for specific target gene expression were standardized with those of the GAPDH gene as the

Table 1. Gene (cDNA) and species-specific primers and conditions used for RT-PCR analysis

Gene (cDNA)	Primer sets	Annealing temperature (°C)	Cycles
CYP1A1	CCATGACCAGGAACTATGGG TCTGGTGAGCATCCAGGACA	60	28
AhR	AGGGAGGTTAAAGTATCTTCATGGAC TCCCTAGGTTTCTCATGATGCTATAC	60	28
ARNT	CTTGGCTCTGTGAAGGAAGG CGGAATCGGAACATGACAG	60	28
inhibin α	TTCATTTTCCACTACTGCCATGGTAGCT GATACAAGCACAGTGTTGTGTAATGAG	55	32
inhibin β-A	TCAACAGTCATTAACCACTACCGCATGA AGCCACACTCCTCCACAATCATGTT	55	34
inhibin β-B	AGGCAACAGTTCTTCATCGACTTTCGGCT AGCCACACTCCTCCACAATCATGTT	55	30
ER α	AATTCTGACAATCGACGCCAG GTGCTTCAACATTCTCCCTCCTC	60	30
ER β	TTCCCGGCAGCACCAGTAACC TCCCTCTTTGCGTTTGGACTA	60	34
AR	CCCATCGACTATTACTTCCCACC TTCTCCTTCTTCCTGTAGTTTGA	60	28
GAPDH	CCCTCAAGATTGTCAGCAATGC GTCCTCAGTGTAGCCCAGGAT	55	24

internal standard.

Statistical analysis: Results are presented as means ± SE. Statistical significance was determined by Student's *t*-test using StatView-J 5.0 software.

RESULTS

Body and testicular weight and size of seminiferous tubules: Body weight was significantly lighter in TCDD-treated rats than in controls at 42 days old (p<0.01). Testicular weight was heavier at those 17 days old and lighter at 42 days old in TCDD-treated rats compared to control rats (p<0.01). The ratio of the testicular weight to body weight was also significantly heavier at 17 days in TCDD-treated rats (p<0.01, data not shown). Morphological examination of testis revealed that seminiferous tubule diameter was significantly greater at 17 days in TCDD-treated rats compared with those of controls (p<0.01) (Table 2). Microscopic observation revealed no significant differences in the numbers of germ cells, Sertoli cells and Leydig cells between TCDD-treated and control rats at either 17 or 42 days (Fig. 1).

Expression of CYP1A1, AhR and ARNT in the liver and testis: CYP1A1 mRNA expression was not observed in the testis of TCDD-treated or control rats at either 17 or 42 days. However, TCDD-treated rats showed a dramatic increase in expression in the liver at 17 days. Compared with control, expression was greater by 10-fold at 17 days and 3-fold at 42 days in the liver of TCDD-treated rats. However, expression of AhR and ARNT mRNA was decreased in the liver of TCDD-treated rats at 42 days compared with controls (Fig. 2).

Expression of steroid receptors: Expression of steroid receptors in the testis of control rats changed with age between 17 days and 42 days. ERα mRNA expression was increased while ERβ mRNA expression was decreased at 42 days. The level of ERα mRNA expression was significantly greater at 17 days in the testis of TCDD-treated rats compared with controls (p<0.01). There was no significant difference in ERα expression in the testis at 42 days between TCDD-treated rats and controls. ERβ expression was greater at 42 days in the testis of TCDD-treated rats, but not at 17 days compared with the control group. TCDD treatment did not alter the level of testicular AR mRNA expression at either 17 or 42 days old (Fig. 3).

Expression of inhibin subunits in the testis: Inhibinα and β-A mRNA expression in the testis of controls decreased with age between 17 and 42 days old. TCDD treatment did not alter the expression of inhibinα, β-A and β-B mRNA at either 17 or 42 days old (Fig. 4).

DISCUSSION

In the present study, we examined the effects of low dose transplacental and lactational exposure of TCDD to male offspring rats. Usually, dams are injected with an initial loading dose of TCDD and a weekly maintenance dose of one fifth of this amount to provide a constant drug concentration in the body, and Faqi *et al.* [6] and Ikeda *et al.* [12] examined the effects of TCDD on male offspring under these conditions. In our experiment, however, 10 *n*g/kg of TCDD was subcutaneously administered to pregnant rats once per week, for a total of 5 times, from gestational Day 7, and reproductive toxicity was evaluated in male offspring. We calculated the deposited TCDD concentration in each dam, as more than 30 *n*g/kg at the final TCDD treatment without consideration of lactational excretion, since it is known that the half-life of TCDD in rats is 2 to 3 weeks [33]. As Nishimura *et al.* [19] demonstrated with the trans-foster experiment, TCDD concentration in serum was more than 10-fold higher in the breast milk-fed group compared with the transplacental-exposed group. Male offspring used in this study were mainly exposed to TCDD from breast milk. Before evaluating the effects of TCDD on the male reproductive system, we first determined CYP1A1 expression in the liver.

We observed that the expression of CYP1A1 mRNA was markedly increased in the liver of TCDD-treated offspring aged 17 days (3 days after final TCDD administration to maternal rats), and increased even in offspring aged 42 days (28 days after final TCDD administration). The reason for the decrease in the expression of AhR and ARNT mRNA in the liver of TCDD-treated rats at 42 days is uncertain at this point. We used TCDD-administrated male rats to evaluate the testicular development and steroid hormone receptor gene expression in relation to low-dose TCDD exposure.

Interestingly, testicular weight was significantly heavier and the ratio of the testicular weight to body weight was significantly greater at 17 days in TCDD-treated rats. Histological examination revealed that, seminiferous tubule diameter was increased in TCDD-treated offspring at 17 days, although there was no difference in the number of

Table 2. The effect of maternal exposure of TCDD on growth and testicular development of male rat offspring

| | 17 days | | 42 days | |
	Control (n=9)	TCDD (n=9)	Control (n=8)	TCDD (n=7)
Body weight (g)	22.2 ± 0.8	23.6 ± 1.2	130.0 ± 2.0	112.9 ± 7.9[##]
Testicular* weight (mg)	58.0 ± 3.1	68.8 ± 5.7[##]	726.0 ± 58.9	597.3 ± 54.8[##]
Diameter of tubules (μm)	92.8 ± 2.6	107.9 ± 4.5[##]	238.3 ± 11.8	230.3 ± 12.3

* Right testicular weight is shown.   ##: p<0.01.

Control   TCDD
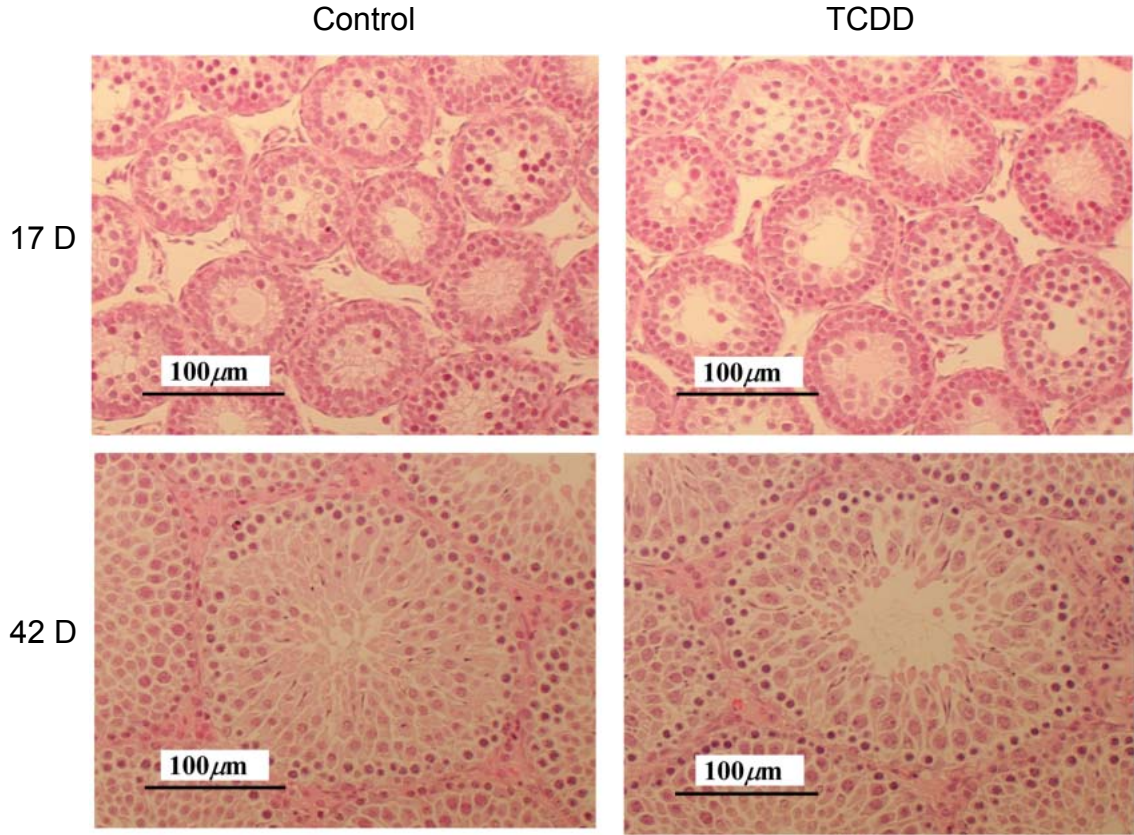
17 D

42 D

Fig. 1.   Microscopic observation of seminiferous tubules in 17-day (17 D)- and 42-day-old (42 D) rats treated with TCDD and
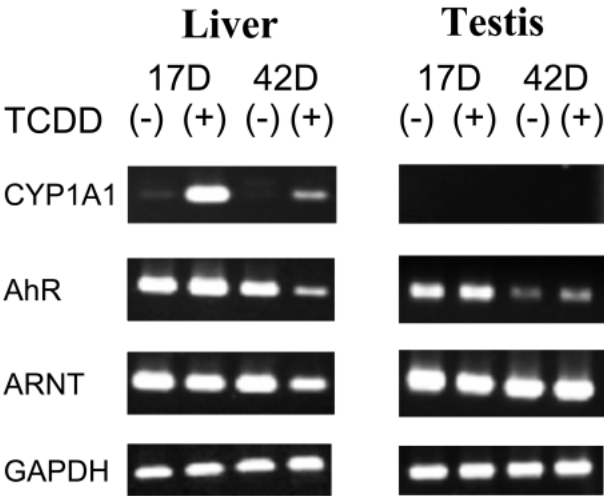control group. The 42 D seminiferous tubules correspond to stage XI. Bar=100 μm (H-E stain, × 132).

Fig. 2.   Semi-quantitative analysis of the gene expression of
CYP1A1, AhR and ARNT cDNA fragments, amplified by RT-
PCR, using testis and liver total RNA of 17-day (17 D)- and 42-
day-old (42 D) TCDD-treated (+) and control (–) rats as tem-
plates. GAPDH expression was used as an internal control.
Images of ethidium bromide stained gel electrophoresis are
shown.

germ cells between TCDD-treated and control animals.
Moreover, expression of ERα was significantly increased in
the testis of TCDD-treated rats at 17 days. These findings
appear to suggest that unusual development of the testis is
induced in TCDD-treated rats during the nursling period.

Many studies have reported that TCDD may cause not
only anti-estrogenic effects but also possible estrogenic
effects, such as induction of endometriosis [10, 27] and the
progression of estrogen-dependent tumors [3, 5]. The role
played by TCDD in ER gene expression is not well known,
however the interactions between ligand-activated AhR and
ER have been investigated [36]. Ohtake *et al.* [22] recently
reported that TCDD-activated AhR/ARNT heterodimer
directly associated with ERα and *β,* and the association
resulted in the recruitment of unliganded ER and a co-acti-
vator for estrogen-responsive gene promoters, leading to
activation of transcription and estrogenic effects. Their
findings suggest that TCDD exerts estrogenic effects under
the absence of estrogen, and anti-estrogenic effects under
the presence of estrogen. We speculate that testicular
growth was accelerated by weak estrogenic effects mediated
by TCDD, since serum estrogen level is lower during the
nursling period [18]. In human, pubertal growth is initiated
by a small increase in estrogen concentration and estrogens
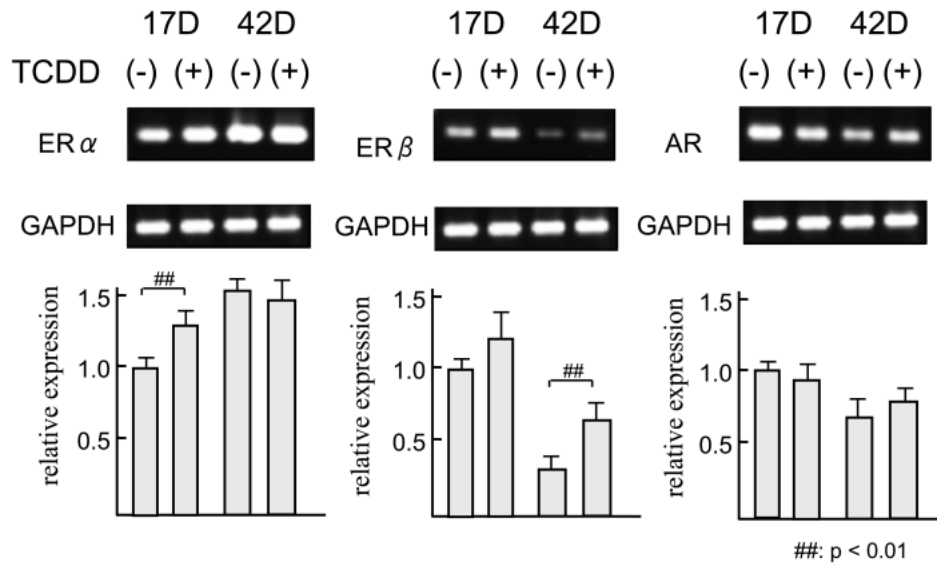promote skeletal construction during sexual maturation. It

Fig. 3. Semi-quantitative analysis of the gene expression of ERα, ERβ and AR cDNA fragments, amplified by RT-PCR, using testis total RNA of 17-day (17 D)- and 42-day-old (42 D) TCDD-treated (+) and control (–) rats as templates. GAPDH expression was used as an internal control. Relative expression is indicated as the ratio to GAPDH expression, and the value of the 17D (–) sample was determined as 1.0. Images of ethidium bromide stained gel electrophoresis are shown.
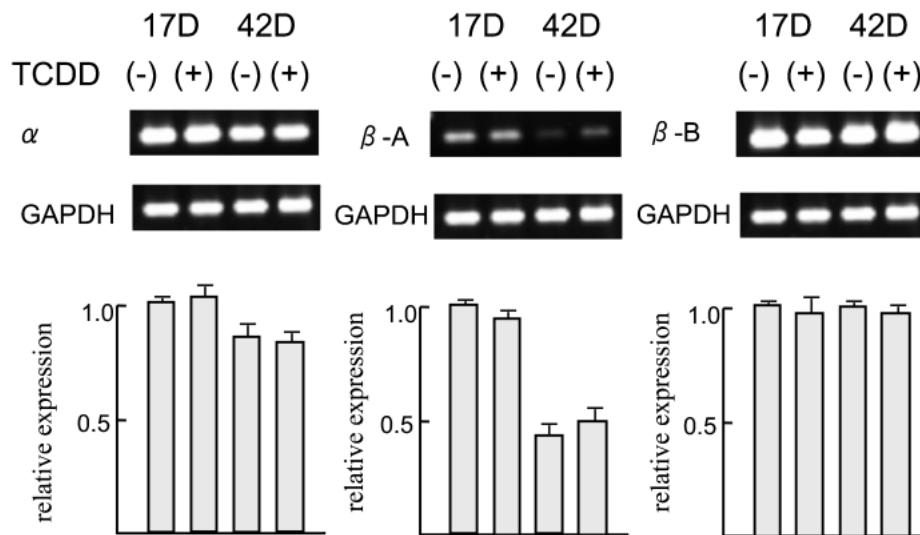

Fig. 4. Semi-quantitative analysis of the gene expression of inhibin subunits (inhibinα, β-A and β-B) cDNA fragments amplified by RT-PCR, using testis total RNA of 17-day (17 D)- and 42-day-old (42 D) TCDD-treated (+) and control (–) rats as templates. GAPDH expression was used as an internal control. Relative expression is indicated as the ratio to GAPDH expression, and the value of the 17D (–) sample was determined as 1.0. Images of ethidium bromide stained gel electrophoresis are shown.

is believed that the weak estrogenic effect of TCDD under the absence of estrogen promote inappropriate testicular growth in premature male rats, and thereafter at 42 days, the anti-estrogenic effect of TCDD under the presence of estrogen and androgen retards skeletal and sexual maturation. Fukuzawa *et al.* [9] reported that TCDD administration significantly reduced the levels of P450scc and luteinizing hormone (LH) receptor in the adult mouse testis. This finding indicates that androgenic effects may be decreased at the time of sexual maturation, resulting in retarded testicular growth. Inhibin is synthesized in Sertoli cells and inhibits FSH synthesis in the pituitary. The expression of inhibin subunits and AR was not significantly changed by TCDD administration.

These results show that the reproductive toxicity of TCDD relates mainly to the estrogen signaling pathway, hence the reason for upregulation of ERβ expression in the 42-day-old TCDD-administered group. Taking these results together, we deduce that the estrogenic and anti-estrogenic effects mediated by low-dose TCDD are complicated and are exerted at various stages of sexual maturation.

In this study, we demonstrated that transplacental and lactational exposure of low-dose of TCDD affects testicular growth and ER gene expression of the testis in offspring. The expression of several genes, such as wings apart-like (WAPL) [14] and dioxin inducible factor-3 (DIF-3) [20], are regulated by TCDD administration. We can evaluate the effects of TCDD on the reproductive system by using these genes as expression standards.

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