Effects of *in utero* 2, 3, 7, 8-Tetrachlorodibenzo-p-Dioxin (TCDD) Exposure on the Reproductive Endpoints of Male Offspring

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The effects of environmental endocrine disruptors (EDs) on the male reproductive system have been receiving much attention, since the male reproductive system is thought to be the system most profoundly affected by EDs. Some researchers have hypothesized that environmental EDs may be responsible for decreased human sperm counts and other male reproductive tract disorders. 2, 3, 7, 8-tetrachlorodebenzo-*p*-dioxin (TCDD) is the most toxic environmental contaminant and well-documented that maternal exposure to TCDD causes irreversible changes in the reproductive system of offspring. The lowest observed effect level (LOEL) of maternal TCDD exposure is much lower than those estimated in the experiments using adult animals. Therefore, the effects of TCDD on human endocrine functions during the developmental stage are a highly important issue in terms of human health risk assessment. Many researchers have reported that *in utero* and lactational exposure to TCDD results in adverse effects on the male reproductive system, i.e., reduced sperm count, reduced size of reproductive organs, and feminized behavior. At the present it is, however, difficult to draw a conclusion concerning the consistent effects of TCDD on testes and sperm production. In this lecture, our recent serial studies using many animal and *in vitro* models would be presented. The aims of our experiments were to investigate the effects of a low dose of TCDD on the male reproductive system, particularly with respect to decreases in testicular size and sperm count, to determine the most sensitive and reliable indexes for risk assessment, and also to study the molecular mechanism for the phenomena.

1. Low dose TCDD experiment for re-evaluation of male reproductive disorders by in utero and lactational TCDD exposure using Holtzman rat model.

Pregnant Holtzman rats were given a single oral dose of 0, 12.5, 50, 200 or 800 ngTCDD/kg on gestational days (GD) 15, and male offspring were sacrificed on several stages after birth. Although there was no TCDD effect in litter size and birth weight of pups for all groups, anogenital distance and ventral prostate weight showed a significant decrease. However there were no changes on testis and epididymal weights, daily sperm production and sperm reserve by TCDD even at the 800 ng/kg dose. To examine androgen dependent prostatic status, the mRNA of 5α-reductase-II (5RD), converting testosterone to dihydrotestosterone (DHT), more potent androgen, was measured. Semiquantitative RT-PCR showed a clear dose-dependent increase unexpectedly. Serum hormone levels, including testosterone, were not changed by any dose

used. Overall there is no good explanation for the season of reduced sex-accessory tissue, just can be said that perinatal TCDD exposure results in decreased androgen sensitiveness in the male offspring.

To investigate the TCDD-induced 5RD up-regulation, cloned 5'-franking region (1713 bp) of 5RD gene was connected to luciferase reporter vector and examined if its transcriptional activity was affected by AhR. Androgen dependent transcription activity in a few cell-lines was also not changed by an increased amount of 3-methyl-cholanthrene. Therefore, it could be concluded that rat 5RD promoter region was not affected by liganded-AhR complex, suggesting that the increase of 5RD expression during postnatal days is not caused by direct interaction TCDD-AhR with 5RD promoter, but that an indirect effect occurred in the ventral prostate of offspring exposed to dioxin *in utero*.

2. Investigation of critical windows concerning *in utero* and lactational TCDD exposure using SD rat model.

Pregnant Sprague-Dawley rats at other developmental stages besides GD15 were exposed to TCDD to compare possible alterations of the male reproductive system. Rats were given a single oral dose of 1 μgTCDD/kg on GD15 or GD18, or male pups born from untreated dams were subcutaneously given a single dose of 1 μgTCDD/kg on PND2. TCDD-exposure on GD15 resulted in significant decreases in urogenital complex and ventral prostate weights and urogenital-glans penis length of male rat offspring on PND70, but not on GD18 and PND2. Testicular and epididymal weights were also lower than control group only in the TCDD-exposed GD15 group. However, reduced testicular size is apparently due to occlusion of corpus epididymis. Anogenital distance was significantly reduced in the TCDD-exposed GD15 and GD18 groups, slightly in the TCDD-exposed PND2 group. These results suggest the presence of a critical window during development with regard to impairments of male reproductive organs by *in utero* and lactational exposure to a low dose of TCDD.

3. Examination of impaired prostate development by using AhR−null mouse.

To examine if the above-mentioned alterations in the male animals are dependent on arylhydrocarbon receptor (AhR) gene or not, here we used AhR knockout mice. Heterozygous (+/−) male and female mice were mated and then injected with 10 μg/kg bw of TCDD on GD13. The AGD of TCDD-treated wild type (+/+) and (+/−) mice offspring on PND14 were significantly decreased 19.6% and 22.2% of the corresponding vehicle control mice, respectively. In contrast, no difference was observed between TCDD-treated homozygous (−/−) mice from control (−/−) mice. Semiquantitative RT-PCR analysis using total RNA from urogenital complex clearly demonstrated that only TCDD-administered (+/+) and (+/−) mice did not express prostatic epithelia specific proteins, probasin, mp25, PSP9 mRNA whereas abundant mRNAs of them was detected in TCDD-treated (−/−) mice as well as all three genotypes from vehicle-treated mice. We thus concluded that alterations in the reproductive system of male offspring maternally exposed to TCDD are dependent on AhR gene.

4. Microarray analysis to detect genes involving in impairment of prostate with the developing urogential sinus of male mouse fetus.

In order to identify genes involved in disruption of prostate development caused by *in utero* TCDD exposure, we administered TCDD to mouse dams during the critical window (GD14) for impairing prostate development, or later during a less TCDD sensitive period (GD17). Microarray techniques were then used to compare gene expression profiles in the urogenital sinus. Authentic biomarker genes for TCDD exposure, such as CYP1A1, CYP1B1, and aryl hydrocarbon receptor repressor (AhRR), were contained in the 15 genes discerned as up-regulated genes both by GD14- and GD17-TCDD treatments, whereas no down-regulated genes were found. Several candidates genes that were up-regulated only by GD14-TCDD included 17β-hydroxysteroid dehydrogenase type II, tumor necrosis factor induced protein 2, and synaptotagmin III, while down-regulated genes included complement factor H or its related proteins. Connexin-30, involucrin, and flavin-containing monooxygenase 5 were also distinguished as candidates genes, the expression of which was significantly up-regulated by GD14-TCDD. Involucrin is a keratinocyte differentiation marker that has been reported to be up-regulated by TCDD exposure. The observed up-regulation of involucrin in UGS suggests that the response of UGS seems to be similar to that in skin tissue. A sort of terminal differentiation of UGS epithelial cells might inhibit the outgr- owth of prostatic buds from the UGS.

5. *In vitro* PCB126 exposure model using the neonatal mouse testis for the analysis of prespermatogenic effect.

3, 3', 4, 4', 5-Pentachlorobiphenyl, one of the polychlorobiphenyl (PCB) congeners, with which can form a planar configuration, has been established to have relatively strong toxicities and has been reported to have the same effect as TCDD on male reproductive system. We investigated the effects of this co-PCB used to study the effects of dioxins on mammalian early spermatogenesis and steroidogenesis in a mouse neonatal testicular organ culture system. Testes collected from newborn mice were cultured in medium containing 0, 10, 100, or 1000 nM co-PCB. Histochemical analysis revealed that the BrdU-labeling indices of both in spermatogenic cells and Sertoli cells were unchanged in all testis specimens exposed to the co-PCB-treated testis. CYP1A1 and steroidogenic enzymes (P450scc, P450c17, 3β-HSD, and 17β-HSD) mRNA levels were measured by semiquantitative RT-PCR. The CYP1A1 mRNA level in cultured testis was significantly increased by co-PCB in a dose-dependent manner. Although mRNA levels of 3β-HSD and 17β-HSD were unchanged, the P450scc mRNA level was significantly down-regulated by co-PCB in a dose-dependent manner. By contrast, the P450c17 mRNA level in 1000 nM co-PCB-treated testis was significantly higher in 1000 nM co-PCB-exposed testis than in control testis. These results suggest that co-PCB does not alter the proliferative activity of spermatogenic cells and Sertoli cells in neonatal testis, but that it directly affects the expression of steroidogenic enzyme genes.

CONCLUSIONS

Perinetal exposure to a relatively low dose TCDD obviously affected on reproductive system in the offspring of experimental animal models used in our serial studies. Anogenital distance and prostate size were the constant endpoints and should be used as criteria to assess dioxin health risk. However, controversy to the previous reports, no effects were observed in testicular function when using the samples in the same experiments, suggesting that spermatogenesis seems to very resistant to perinatal dioxin exposure. It should be given a careful consideration to accept the hypothesis that dioxins, the most potent endocrine disruptors, impairs sperm counts by affecting on male germ cell development and then reduce fertility in human population.

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