mal cells of the urogenital sinus [34] and was not caused by interruption of androgen signaling in this tissue [35]. It is still unclear how TCDD impairs the responsiveness of the developing prostate to androgen.

In Utero and Lactational TCDD Exposure Decreased Androgen Responsiveness of the Prostate

In the histological examinations, the prostatic lobes developed with obvious epithelial layers in TCDD-exposed $Ahr^{+/+}$ mice, indicating TCDD-exposed prostates may function as normal exocrine glands (Fig. 2, B and C). However, using RT-PCR and in situ hybridization analyses, we could not detect prostatic epithelial secretory protein mRNAs in the TCDD-exposed UGCs (Figs. 1 and 3). Fewer layers of epithelial cells and increased cell numbers in mesenchymal cells were observed in the TCDD-exposed $Ahr^{+/+}$ animals, suggesting that in utero and lactational exposure to TCDD produced functional abnormalities (Fig. 2, E and F). The prostatic secretory proteins, PBSN, MSMB, and SBP, were reportedly upregulated via the androgen receptor [36–38]. Since no significant differences in intratesticular testosterone levels were found between control and TCDD-exposed animals on PND14, we speculated that abnormal development of prostate glands may be due to decreased androgen sensitivity or that TCDD disrupts mouse prostate epithelial cell differentiation into luminal epithelial cells. This notion is consistent with a previous study [9] in the sense that the ventral prostate of male rat offspring exposed to TCDD in utero and via lactation did not respond to the exogenous androgens testosterone, 5α-dihydrotestosterone (DHT), and 5α-androstane-3α, 17β-diol, in the organ culture system. Administration of the androgen receptor antagonist flutamide and the 5α-reductase inhibitor finasteride to rats in the late pregnancy period did not cause prostate growth in male offspring on PND60 [39], suggesting that DHT is an essential steroid hormone for prostate development. Since the inhibition was found specifically in the prostate but not in the seminal vesicle, the reduction in DHT production in the prostate was initially hypothesized to occur in males exposed to TCDD in utero and via lactation. However, 5α-reductase type 2 enzymatic activity and mRNA expression was elevated, compared to control groups [9, 10]. Thus, it is reasonable to speculate that decreased androgen responsiveness in the TCDD-exposed offspring was caused by other factors. Our previous study using Holtzman rats showed significantly reduced androgen receptor mRNA expression in the ventral prostate following in utero and lactational TCDD exposure, suggesting that the decreased androgen responsiveness might be due to reduced amounts of receptor molecules [10]. However, we could not detect a significant reduction in androgen receptor mRNA here, probably due to a difference in animal species or organs used for RT-PCR analysis.

Function of AHR in the Testis

AHR was reportedly responsible for apoptotic signaling, and the number of primordial cells in the ovarian germ line was not attenuated due to a defect in the apoptosis in *Ahr*-null female mice [20]. More recently, testicular dysfunction was reported in aged *Ahr*-null mice [40], and HSD3B1 expression in Leydig cells was significantly reduced at 24 wk, resulting in serum testosterone decline, lowered sperm number, and reduced size of seminal vesicles. AHR seems to play a role in maintaining normal steroidogenesis in aged animals. However, in that study, there were no significant differences in testicular functions between wild-type and *Ahr*-null mice during younger stages (10 wk old). In our present study, we were unable to find differences in testicular functions among the *Ahr* genotypes, including testosterone production, Sertoli cell differentiation, and spermatogenic cell differentiation. Therefore, it is reasonable to conclude that AHR has very little function in early stages of gonad development. If the AHR functions even at the early stage of development, the functional redundancy with other genes may also exist among *Ahr* and other genes during testicular development.

Resistance of Testicular Development to In Utero and Lactational TCDD Exposure

In our previous study, TCDD administration to pregnant Holtzman rats on GD15 did not alter the testicular weight or serum testosterone levels of male offspring on PND49 [10]. In our present study, we did not find any differences in testicular cell differentiation levels between control *Ahr*-carrying animals and TCDD-exposed animals that survived the high dose of TCDD exposure, including supporting cell marker and spermatogenic cell differentiation markers, which suggested that even in the TCDD-exposed animals testicular differentiation proceeded normally. 5α-androstane-3α, 17β-diol, the major form of testicular androgen in immature mice [32], was previously reported to be slightly reduced in PND21 mice perinatally exposed to TCDD [17]. However, in our present study, in the assayed testes from PND14, no difference was seen in both *Akr1c4* and *Srd5a1* mRNA expression levels among the three genotypes and TCDD treatment. Moreover, in utero and lactational exposure to TCDD did not alter the expression levels of steroidogenic enzyme genes in Leydig cells in $Ahr^{+/+}$ and $Ahr^{+/-}$ animals. At a relatively high dose, testicular CYP11A1 activity was reduced by TCDD exposure [41]. In our previous report, we also found that administration of 100 μg TCDD/kg to adult male mice reduced testicular *Cyp11a1* mRNA and protein levels [42], and in vitro co-planer PCB (3,3′,4,4′,5-pentachlorobiphenyl; PCB126) exposure to neonatal mouse testis downregulated *Cyp11a1* mRNA expression [33]. The reason why we could not detect the reduction in *Cyp11a1* mRNA in the present study is not clear, but it is speculated that intratesticular levels of TCDD in male pups born to dams given TCDD on GD13 was not sufficient to downregulate *Cyp11a1* by PND14.

Although testes and UGCs were collected from the same individual pups, both *Cyp1a2* and *Cyp1b1* mRNAs were not induced in the testes of mice with any of the three genotypes, whereas in the UGCs, *Cyp1a2* and *Cyp1b1* were significantly induced. Moreover, an approximately 30-fold increase in *Cyp1a1* was observed in the $Ahr^{+/+}$ UGCs. Thus, UGCs are much more sensitive to TCDD than testis in terms of *Cyp1a1* mRNA induction. Using a xenobiotic-responsive element connected to the β-galactocidase reporter gene, a transgenic mouse line was generated and then exposed to TCDD in utero and via lactation [43]. X-Gal staining analysis clearly demonstrated that fetal urogenital tracts showed significant induction of the reporter gene, but that the testis did not respond. Based on the above-mentioned results, we speculated that UGCs express modulating factors (modifiers) that enhance AHR-mediated transcription, and that these were lacking or present in small amounts in the testis. Although it cannot be excluded that tissue concentration of TCDD in the testis was lower than that in the UGC, it is more likely that the testis is much more resistant to TCDD exposure than the UGC.

In conclusion, using *Ahr* knockout mice, we confirmed that in utero exposure to TCDD caused *Ahr*-dependent impairment of prostate development and reduced anogenital distances in

male offspring, but that the testicular development seemed to be resistant to TCDD exposure. AHR was not associated with testicular development under physiological conditions.

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RESEARCH

Induction of spermatogenic cell apoptosis in prepubertal rat testes irrespective of testicular steroidogenesis: a possible estrogenic effect of di(*n*-butyl) phthalate

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Abstract

Although di(*n*-butyl) phthalate (DBP), a suspected endocrine disruptor, induces testicular atrophy in prepubertal male rats, whether it exerts estrogenic activity *in vivo* remains a matter of debate. In the present study, we explored the estrogenic potency of DBP using 3-week-old male rats, and then examined the relationship between estrogen-induced spermatogenic cell apoptosis and testicular steroidogenesis. Daily exposure to DBP for 7 days caused testicular atrophy due to loss of spermatogenic cells, whereas testicular steroidogenesis was almost the same with the control values. A single exposure of DBP decreased testicular steroidogenesis in addition to decreasing the level of serum LH at 3 h after DBP treatment, with an extremely high incidence of apoptotic spermatogenic cells at 6 h after administration. To elucidate the estrogenic activity of DBP, we carried out an inhibition study using pure antiestrogen ICI 182,780 (ICI) in a model of spermatogenic cell apoptosis induced by DBP or estradial-3-benzoate (EB). Although both the DBP- and EB-treated groups showed a significant increase in spermatogenic cell apoptosis, ICI pretreatment significantly decreased the number of apoptotic spermatogenic cells in these two groups. In contrast, testicular steroidogenesis and serum FSH were significantly reduced in all the treated groups, even in the DBP+ICI and EB+ICI groups. Taken together, these findings led us to conclude that estrogenic compounds such as DBP and EB induce spermatogenic cell apoptosis in prepubertal rats, probably by activating estrogen receptors in testis, and that reduction in testicular steroidogenic function induced by estrogenic compounds is not associated with spermatogenic cell apoptosis.
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Introduction

Di(*n*-butyl) phthalate (DBP) is one of the most widely studied phthalate esters that disrupt the growth of normal reproductive organs, because of its wide use as a plasticizer in cosmetics, printing inks, and pharmaceutical coatings. The most prominent effect of DBP is testicular atrophy (Oishi & Hiraga 1980, Gray *et al.* 1982). To date, several mechanisms have been proposed to explain the induction of testicular atrophy by DBP, such as the depletion of zinc (Oishi & Hiraga 1980), increased oxidative damage of proteins, lipids, and DNA (Fukuoka *et al.* 1990), alteration of vimentin cytoskeleton organization (Kleymenova *et al.* 2005), or membrane alteration in Sertoli cells leading to sloughing of spermatogenic cells (Kleymenova *et al.* 2005). However, the cellular target of DBP and molecular mechanisms of DBP-induced spermatogenic cell apoptosis remain to be unknown.

The study on serum levels of DBP in thelarche patients (a premature breast development before age 8) showed that 28 of 41 (68%) thelarche patients displayed significantly higher (15–276 mg/l) levels of DBP (Colon *et al.* 2000). The high-serum level of DBP in thelarche patients is a matter of concern as an estrogenic xenobiotic. DBP and butyl benzyl phthalate were found to be capable of binding to estrogen receptor α (ERα) and then enhancing the proliferation of MCF-7 human breast cancer cells expressing ERα (Jobling *et al.* 1995, Harris *et al.* 1997, Zacharewski *et al.* 1998, Nishihara *et al.* 2000). In addition, DBP has been shown to exhibit an

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estrogenicity in an E-screen assay (Soto *et al.* 1995, Hong *et al.* 2005) and to prevent tamoxifen, an ER antagonist, from inducing apoptosis in MCF-7 cells (Kim *et al.* 2004). In contrast, phthalate esters including DBP displayed no biological activity in a rat uterotrophic assay, an *in vivo* screening test for estrogenicity (Milligan *et al.* 1998, Zacharewski *et al.* 1998). Therefore, the specific cellular target of DBP is still a matter of debate.

The role of estrogen in spermatogenesis has been reported that estrogen is essential for spermatogenesis (Eddy *et al.* 1996, O'Donnell *et al.* 2001), spermatogenic cells express ERs (Saunders *et al.* 1998, O'Donnell *et al.* 2001), and estrogen-like chemicals present in the environment adversely affect male reproductive health (Akingbemi & Hardy 2001). Such chemicals have the ability to affect gene expression and cellular function by binding to hormone receptors (Hall & Korach 2002). It is generally accepted that the hypothalamo-pituitary–gonadal (HPG) axis regulates spermatogenesis by controlling circulating levels of LH through the feedback regulation of steroid hormones, and this feedback loop can be intercepted by endocrine-disrupting chemicals (EDCs) binding to ERs in hypothalamus. Direct interference to spermatogenic cells is also possible, because these cells are known to express ERs (Saunders *et al.* 1998, 2002). Adult male hamsters given diethylstilbestrol showed a significant decrease in gonadotropin levels, leading to the increased spermatogenic cell apoptosis through the suppression of testosterone level (Nonclercq *et al.* 1996). In another study, administration of a single high dose of estradial-3-benzoate (EB) to 1-day-old male rats causes a reduction in GnRH secretion, resulting in the suppression of circulating level of LH and consequently testosterone (Tena-Sempere *et al.* 2000). Estrogen is directly involved in the suppression of testicular ER expression (Tena-Sempere *et al.* 2000). Therefore, we hypothesize that ERs present in testes may have a role in the induction of spermatogenic cell apoptosis by estrogenic compounds.

ICI 182,780 (ICI), a pure estrogen antagonist, binds to both ERα and ERβ (Kuiper *et al.* 1998, Howell *et al.* 2000), does not cross the blood–brain barrier (Wade *et al.* 1993), and is currently used as a first choice treatment for human breast cancer, because of its local peripheral action (Wiebe *et al.* 1993). In the present study, in order to reveal the estrogenicity of DBP, attempts were made to block the ERs on prepubertal rat testes with ICI prior to DBP or EB administration.

Results

Effects of repeated DBP exposure on testis morphology and testicular steroidogenesis

Daily administration of DBP for 7 days resulted in a significant decrease of testis weight in a dose-dependent manner (Fig. 1A). In histological observations, the most common lesion site was a seminiferous tubule. Lesions in seminiferous tubules were characterized by decreased tubular size, depletion of spermatogenic cells, wider tubular lumen, and ultimately a thin layer of seminiferous tubules depending on the dose. Detachment of spermatogenic cells, reduction in diameter of tubular size, and giant cells with cellular debris in tubular lumen were frequently observed in the group treated with 500 mg/kg per day DBP (Fig. 1D), while complete loss of spermatocytes in seminiferous tubules was seen in the group treated with 1000 mg/kg per day DBP, leading to irregularly shaped and sized tubules (Fig. 1E). We performed a TUNEL assay to examine whether degraded spermatogenic cells were undergoing apoptosis. A significant number of apoptotic (TUNEL-positive) spermatogenic cells were detected in the 500 mg/kg per day group (Fig. 1H) as compared with the control (Fig. 1F). In the 1000 mg/kg per day group, only a few apoptotic spermatogenic cells were found due to the complete loss of spermatocytes (Fig. 1I). The number of apoptotic cells per seminiferous tubule was higher in the 500 mg/kg per day group than in the 250 mg/kg per day group.

To examine the possible involvement of testicular steroidogenesis in spermatogenic cell apoptosis, the ultrastructure of Leydig cells was observed by transmission electron microscopy, and intratesticular testosterone (ITT) levels were analyzed by testosterone EIA assay. We selected 500 mg DBP/kg per day as a representative dose, based on the above observation. Several lipid droplets in the Leydig cell cytoplasm were distinctly observed in the treated group, whereas these droplets were not found in the control group (Fig. 2A and B). The average ITT concentration of the DBP-treated groups was almost the same with that of the control group (Fig. 2C). The mRNA expression levels of four steroidogenic enzymes were also analyzed by real-time RT-PCR. Although statistical significance was not detected, DBP exposure apparently increased the cytochrome P450 side chain cleavage (*P450scc, Cyp11a1*) and 3β hydroxysteroid dehydrogenase (*3βHsd, Hsd3b*) expressions (Fig. 2D and F). The cytochrome P450 17α/C_{17-20} lyase (*P450c17, Cyp17a1*) and 17β hydroxysteroid dehydrogenase (*17βHsd, Hsd17b*) expressions in the DBP-treated groups were almost the same with that of the control group (Fig. 2E and G). These findings indicate that the testes after daily exposure of DBP for 7 days shows normal or slightly enhanced steroidogenesis with a high rate of seminiferous tubule disruption.

Effects of an acute single exposure of DBP on spermatogenic cell apoptosis and testicular steroidogenesis

In a 7-day daily exposure of DBP, seminiferous tubules showed a depletion of spermatogenic cells, probably due to apoptosis. In order to determine the exact time point of maximum apoptosis and the exact target cell

type for DBP insult, we conducted a time-course study with exposure times of 3, 6, and 24 h after a single exposure of DBP. We found a significant increase in the number of apoptotic spermatogenic cells in the treated groups in comparison with the control (Fig. 3A–D). The maximal number of apoptotic spermatogenic cells was detected at 6 h after treatment (Fig. 3C and J). At 24 h after administration, the number of apoptotic cells began to gradually decline, although it was still significantly greater than that in the control group (Fig. 3J).

In order to evaluate the spermatogenic cell types that underwent apoptosis, apoptotic cells were analyzed by light and transmission electron microscopies. At the light microscopic level, apoptotic cells were identified by their prominent basophilia and shrinkage of both cytoplasm and nucleus. Spermatocytes were the largest cells and showed typically dispersed chromatin, and spermatogonia were identified by their location (Fig. 3F and G). Similarly, at the electron microscopic level, apoptotic spermatocytes with chromatin clumping and shrinkage of cytoplasm and nucleus were clearly distinguishable (Fig. 3H and I).

ITT level was also measured by a testosterone EIA assay. In contrast to 7-day daily exposure, a single exposure of DBP (500 mg/kg) significantly decreased the level of ITT (Fig. 4A). To address whether DBP suppressed the ITT level by affecting the HPG axis, we analyzed the serum LH level by RIA. Inhibition of testicular testosterone production by estrogen administration is mediated by a decrease in serum LH level through the action of estrogen on the hypothalamo-pituitary axis (Jong *et al.* 1975). As shown in Fig. 4B, the serum LH concentration showed a tendency to decrease ($P=0.06$) in the DBP-treated groups, especially at 3 h after DBP administration. These data suggested that DBP might have blocked LH secretion shortly after administration, probably due to estrogenic activity in hypothalamus, and then diminished the stimulation of Leydig cells, thereby reducing the production of testosterone (Fig. 4). Real-time RT-PCR analysis also showed a statistically significant reduction in *Cyp11a1*, *Cyp17a1*, *Hsd3b*, and *Hsd17b* expressions by DBP treatment (Fig. 4C–F). The decrease in ITT nearly paralleled the decrease in the mRNA levels of all testicular steroidogenic enzymes (*Cyp11a1*, *Cyp17a1*, *Hsd3b*, and *Hsd17b*). These results illustrated that DBP decreased testicular steroidogenesis and that this decrease likely occurred via suppression of the HPG axis, suggesting that DBP acts as an estrogen agonist.

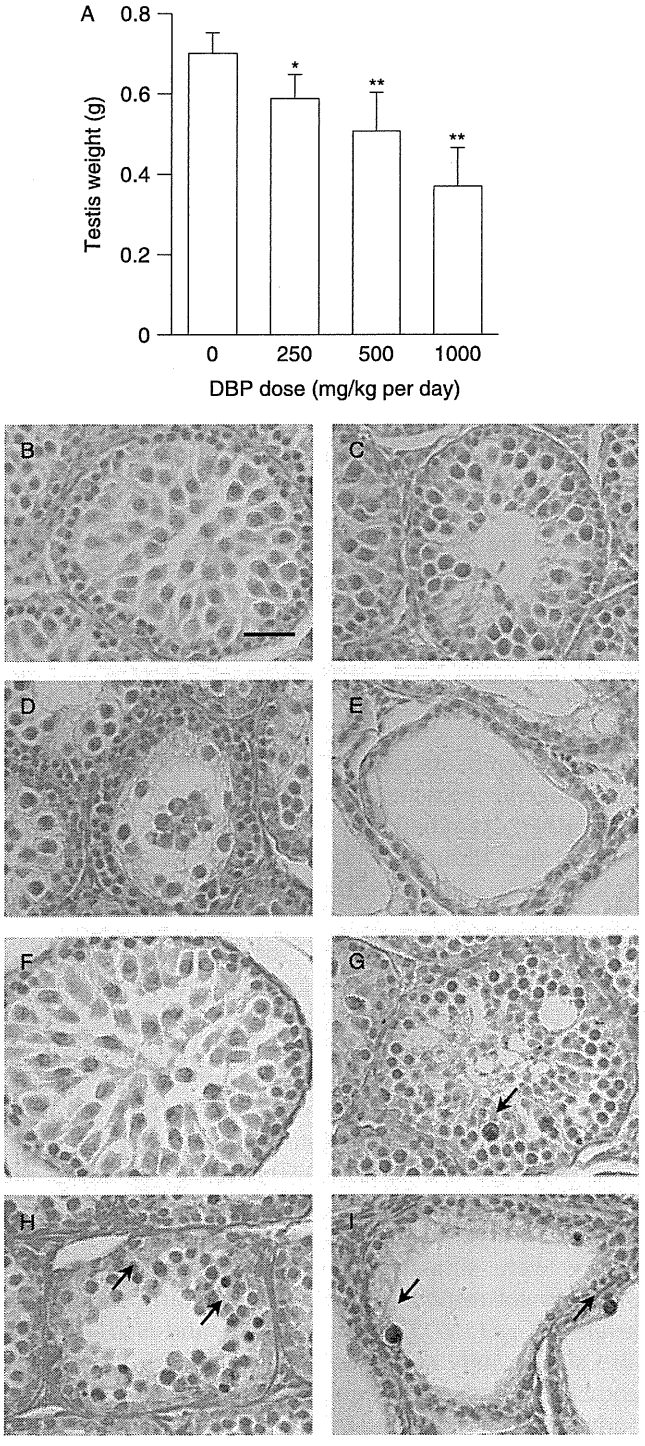

Figure 1 Effects of repeated DBP exposure on testis morphology. Testis weight after once daily administration of DBP for 7 days (A) is shown. Results are expressed as the means±S.E.M. ($n=6$). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*$P<0.05$; **$P<0.01$ versus control). Histological changes of testes after once daily administration of DBP for 7 days are shown using hematoxylin and eosin staining (B–E) and TUNEL labeling (F–I). Results are shown for the vehicle-treated (B), 250 mg/kg per day (C), 500 mg/kg per day (D), and 1000 mg/kg per day (E) groups. Note the reduction in diameter and irregularly shaped seminiferous tubules due to depletion of spermatogenic cells. TUNEL-positive (apoptotic) spermatogenic cells are indicated by arrows in testes. Vehicle-treated control (F), 250 mg/kg per day (G), 500 mg/kg per day (H), and 1000 mg/kg per day (I) treated groups. Scale bar, 20 μm.

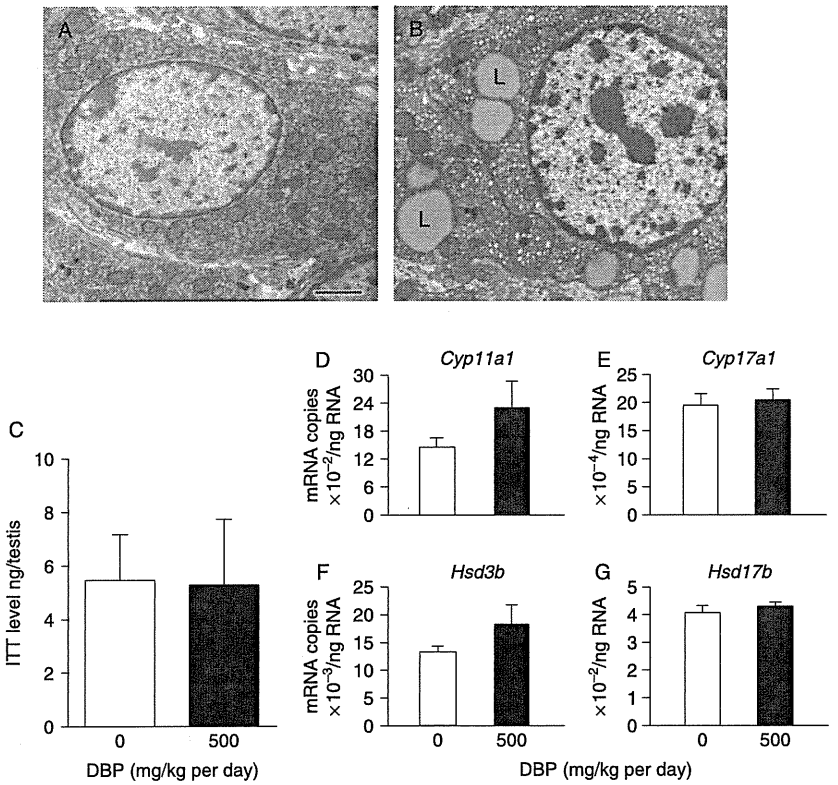

Figure 2 Effects of repeated DBP exposure on testicular steroidogenesis. Ultrastructural changes in Leydig cells of the control (A) and 500 mg/kg per day treated groups (B). L indicates lipid droplets. Scale bar, 1 μm. Intratesticular testosterone (ITT) level (C) and testicular mRNA expressions for *Cyp11a1* (D), *Cyp17a1* (E), *Hsd3b* (F), and *Hsd17b* (G). ITT level was measured by testosterone EIA assay, and mRNA expressions of testicular steroidogenic enzymes were determined by real-time RT-PCR. Results are expressed as the means±S.E.M. ($n=8$ for ITT and $n=3$ for testicular steroidogenic enzymes mRNA expressions). Statistically significant differences between means from the control and treated groups were determined by Student's *t*-test.

Antiestrogen ICI inhibited DBP- or EB-induced spermatogenic cell apoptosis

A single exposure of DBP resulted in decreased testicular steroidogenesis by suppression of LH level together with increased spermatogenic cell apoptosis. To examine the hypothesis that spermatogenic cell apoptosis is induced by estrogenic compounds through ERs, we further conducted an inhibition study with pure antiestrogen ICI. The effect of EB, a synthetic long-term acting estrogen which is converted to 17β-estradiol (E_2) in liver, was examined using male rats of the same age. As shown in Fig. 5B, 500 mg/kg of DBP significantly increased ($P<0.001$) the number of apoptotic spermatogenic cells compared with that in the control. Treatment with 200 μg/kg EB also significantly increased ($P<0.001$) the number of apoptotic spermatogenic cells as compared with that in the control group, although the number of apoptotic spermatogenic cells in the EB-treated group was lower than that in the DBP-treated group (Fig. 5D). In the rats treated with the above doses of DBP and EB, pretreatment with ICI decreased ($P<0.001$) the apoptotic spermatogenic cell number compared with that in the groups without pretreatment (Fig. 5C, E, and G).

Comparison of the effects of DBP and EB on testicular steroidogenesis and serum FSH level

In order to examine the possible correlation between inhibition of DBP- or EB-induced spermatogenic cell apoptosis by ICI and testicular steroidogenesis, the testicular steroidogenic capacity was examined in the samples obtained from the above inhibition study (Fig. 5). It is known that testosterone withdrawal results in spermatogenic cell apoptosis (Blanco-Rodriguez & Martinez-Garcia 1997, 1998, Creasy 2001, McLachlan *et al.* 2002). In our study, a single exposure of DBP caused a significant reduction in ITT as well as testicular steroidogenic enzymes expressions (Fig. 4). Therefore, we further examined whether ICI pretreatment would inhibit the reduction in testicular steroidogenesis induced by DBP or EB. However, ITT level was significantly lower in the DBP+ICI− ($P<0.001$) or EB+ICI− ($P=0.001$) treated group compared with that in the intact and control groups. The level in the pretreated groups was similar to that in the group treated with DBP ($P<0.001$) or EB ($P<0.001$) alone (Fig. 6A). The serum FSH level was also measured in the samples obtained from the above inhibition study (Fig. 5) to correlate between the apoptotic index and testicular steroidogenesis (Figs 5 and 6). The serum FSH level was significantly decreased in the DBP- ($P<0.001$) and EB- ($P<0.001$) treated groups. These levels in the pretreated groups were similar to those in the groups without pretreatment respectively (Fig. 7). Similarly, the mRNA levels of *Cyp11a1*, *Cyp17a1*, *Hsd3b*, and *Hsd17b* were also significantly decreased in the DBP, EB, DBP+ICI, and EB+ICI groups compared with those in the control and intact groups (Fig. 6B–E), whereas no significant

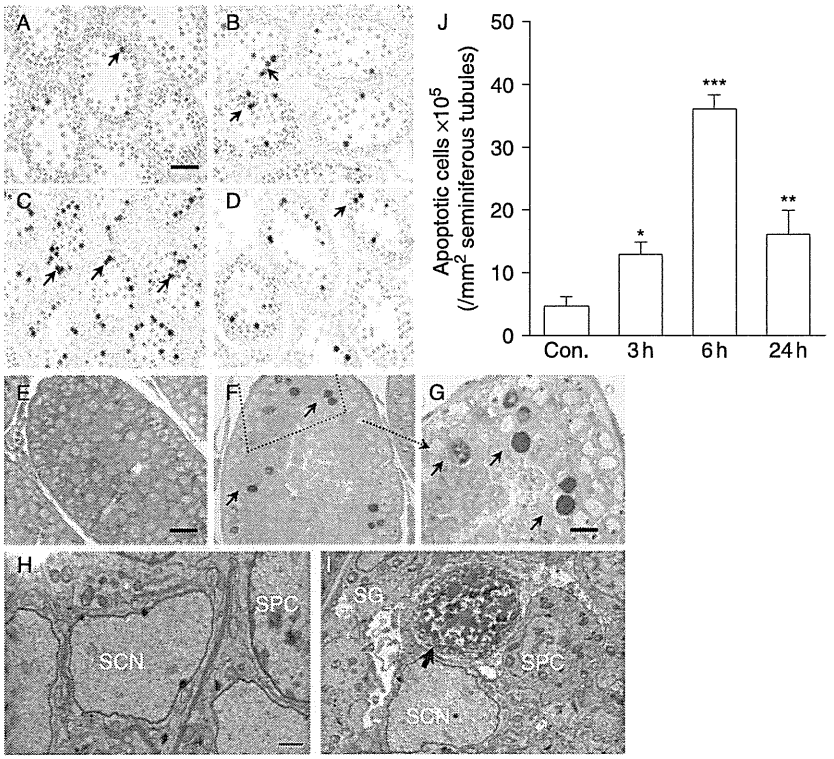

Figure 3 Spermatocytes apoptosis after a single exposure of 500 mg DBP/kg. TUNEL labeling of testes. Vehicle-treated control (A), and at 3 h (B), at 6 h (C), and at 24 h (D) after treatment. Note the maximal apoptotic spermatogenic cell number at 6 h after treatment. Arrows indicate apoptotic spermatocytes. Scale bar, 50 μm. Semi-thin sections of seminiferous tubules of the control (E), and at 6 h (F and G) after treatment. Toluidine blue staining. E–F, scale bar, 20 μm. G, scale bar, 10 μm. Transmission electron micrographs of control (H) and at 6 h (I) after treatment. Arrowhead, apoptotic spermatocyte; SCN, Sertoli cell nucleus; SG, spermatogonium; SPC, spermatocyte. Scale bar, 1 μm. Quantification of apoptotic spermatogenic cells (J). Values representing the number of apoptotic cells per 1 mm^2 seminiferous tubules areas are expressed as the means±S.E.M. (n=8). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*P<0.5, **P<0.01, ***P<0.001 versus control).

changes of ITT concentration, serum FSH level, and steroidogenic enzymes expressions were found in the ICI alone-treated group compared with those in the intact and vehicle groups.

Discussion

Repeated DBP exposure causes testicular atrophy without significant changes in testicular steroidogenesis

The present study demonstrated that repeated administration of DBP for 7 days increased the number of disrupted seminiferous tubules with spermatogenic cell depletion. Disturbance in seminiferous tubules and enhanced testicular steroidogenesis have been reported in prepubertal rats chronically exposed to DBP (Ryu *et al.* 2007). In our study, however, there was no significant change in testicular steroidogenesis, although excess lipid droplets were clearly observed in Leydig cells. The discrepancy in testosterone level between this study and Ryu *et al.* (2007) may be due to the difference in exposure period (7 vs 30 days). Our study, however, showed a similar level of disrupted seminiferous tubules to that by Ryu *et al.* (2007), indicating that testosterone production may not be associated with testicular atrophy induced by DBP.

Acute DBP exposure increases spermatogenic cell apoptosis and decreases testicular steroidogenesis

It is well established that testosterone synthesis is controlled by negative feedback regulation of HPG axis, and an increase in testosterone level causes a reduction in LH secretion from pituitary by activating androgen receptor (AR) in hypothalamus. A testosterone metabolite by aromatization, E_2, can also reduce LH secretion by binding to ERs in hypothalamus as well as androgens. Then, a subsequent reduction in LH pulse would lead to a suppression of testosterone production. Decreased testicular testosterone biosynthesis as well as decreased serum LH level and serum FSH level occurs after exogenous estrogen exposure, together with increased spermatogenic cell apoptosis (D'Souza *et al.* 2005). To our knowledge, the present study reveals for the first time that DBP suppresses testicular steroidogenesis with a correspondent decrease in serum FSH (Fig. 7) and serum LH level (Fig. 4B) shortly after treatment, probably by interacting with ERs in hypothalamus. In addition, the similar reduction in serum FSH level was found in the EB-treated group (Fig. 7). All of the data indicate that phthalate ester has an estrogenicity similar to that of E_2.

It is well established that testosterone synthesis depends on the frequency and amplitude of LH pulse, and disruption of LH pulse by administration of estrogen has been shown to cause the suppression of testosterone

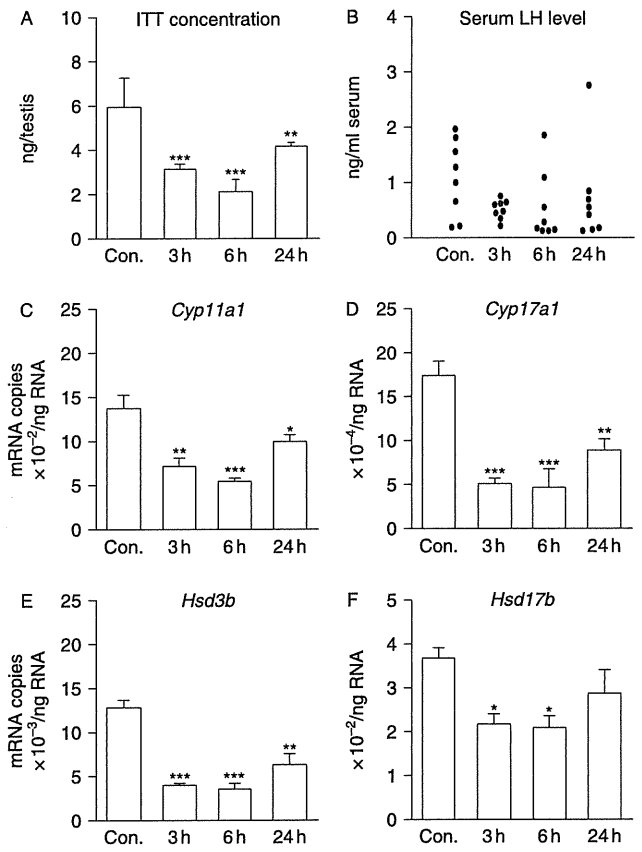

Figure 4 Testicular steroidogenesis and serum LH levels after a single exposure of 500 mg DBP/kg. Intratesticular testosterone (ITT) level (A), serum LH level (B), and testicular mRNA expressions for *Cyp11a1* (C), *Cyp17a1* (D), *Hsd3b* (E), and *Hsd17b* (F). ITT level was measured by testosterone EIA assay, serum LH was measured by RIA, and mRNA expressions of testicular steroidogenic enzymes were analyzed by real-time RT-PCR. Note that the decreased serum LH pulse led to a reduction in ITT level as well as a decrease in mRNA expressions in the treated rats. Results are expressed as the means ± S.E.M. ($n=8$ for ITT and serum LH, and $n=3$ for real-time RT-PCR). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*$P<0.05$; **$P<0.01$, ***$P<0.001$ versus control).

production (McGarvey *et al.* 2001). To demonstrate the effects of DBP on LH pulsatility, the serial blood sampling at several minute intervals from live animals and the appropriate pulse analysis should be required. But, this is difficult to detect in the present study because of a single spot blood sample. DBP might induce disruption of LH pulse and subsequently decrease testicular testosterone production. On this point, further studies are required in the future.

DBP-induced spermatogenic cell apoptosis is mediated via ERs

There is much evidence that DBP can interact with ERs. For example, several studies employing an ER-mediated yeast growth assay or a reporter assay with human breast cancer cell line MCF-7 have demonstrated the interaction between DBP and ERs (Jobling *et al.* 1995, Harris *et al.* 1997, Zacharewski *et al.* 1998, Andersen *et al.* 1999). Moreover, DBP exhibited an estrogenicity in an E-screen assay (Soto *et al.* 1995) and prevented tamoxifen (ER antagonist)-induced apoptosis in MCF-7 cells (Kim *et al.* 2004). However, it is still unclear whether DBP has an estrogenicity in an *in vivo* experimental model, because any phthalate esters, including DBP, displayed no significant changes in the female reproductive organ weight in the rat uterotrophic assay (Milligan *et al.* 1998, Zacharewski *et al.* 1998). In the present study, decreased testicular steroidogenesis was observed in a single exposure of DBP with the same spectrum of those of the EB-treated groups (Fig. 6). Administration of synthetic estrogens, including EB, is known to induce spermatogenic cell apoptosis. Here, for the first time, we have demonstrated that pure antiestrogen ICI inhibited EB-induced spermatogenic cell apoptosis. Similarly, DBP-induced spermatogenic cell apoptosis was significantly decreased by ICI pretreatment (Fig. 5), clearly demonstrating that, at least in immature male rats, DBP acts as an estrogen agonist. To our knowledge, this is the first report using an *in vivo* model to demonstrate the estrogenicity of DBP through ERs.

EB- and DBP-induced spermatogenic cell apoptosis was blocked by pretreatment with an ER antagonist, ICI, indicating that E_2 as well as DBP was functioning through ERs. Indeed, both receptors are present in testes (Saunders *et al.* 1998, Pelletier *et al.* 2000). In general, the classic ER-signaling pathway involves the binding of the ligand-bound ERs (either α or β) to the estrogen-responsive element (ERE) that regulates transcription of target genes. However, ERs also mediate gene transcription by binding to an AP-1 element together with the transcription factors, Fos and Jun (McEwen & Alves 1999). ERα and ERβ have been shown to transcript in opposite ways from the AP-1 site; when bound to E_2, ERα activates and ERβ inhibits transcription (Paech *et al.* 1997). In contrast to the endogenous estrogen, antiestrogens, including raloxifene, and ICI have been shown to effectively activate transcription from an AP-1 site when bound to ERβ (Paech *et al.* 1997). The intracellular mechanisms of ERs action in the present study and the possible involvement of both ERα and ERβ in spermatogenic cell apoptosis are not known, and further studies are required. More recent work has been shown that estrogen action in male reproductive system does not involve EREs pathway (Weiss *et al.* 2008). Moreover, many studies revealed a nongenomic signaling pathway through membrane-associated ERs (Hammes & Levin 2007) and cross-talk between genomic pathways (Revelli *et al.* 1998, Losel *et al.* 2003). Binding of E_2 to the membrane-associated ERs results in rapid (within minutes) activation of the MAP kinase, phosphatidylinositol 3-kinase, and protein kinase C and phosphatases, as well as the release of several cyclic amines

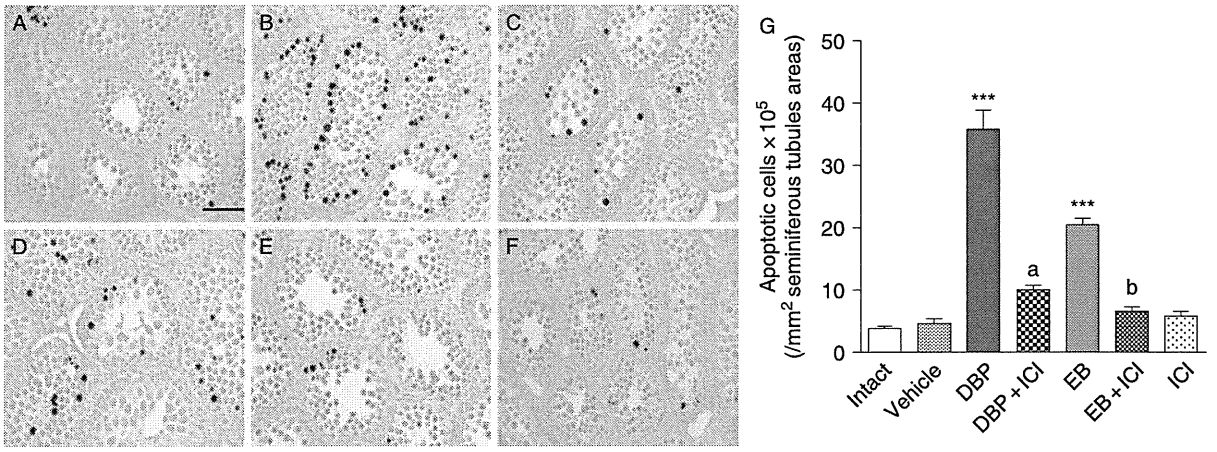

Figure 5 ICI inhibits DBP- or EB-induced spermatogenic cell apoptosis. Results are shown for the control (A), DBP alone (B), DBP+ICI (C), EB alone (D), EB+ICI (E), and ICI alone (F) groups. Rats ($n=12$) were i.p. administered with 3 mg/kg of ICI (5% ethanol and 95% corn oil) 4 h prior to administration of DBP or EB. The doses of DPB and EB were 500 mg/kg and 200 μg/kg respectively. Apoptotic spermatocytes are stained brown. Scale bar, 50 μm. Comparison of the numbers of TUNEL-positive cells in the testes of treated rats (G). Note that the numbers of both DBP- and EB-induced apoptotic spermatocytes were decreased by ICI pretreatment. Values representing the number of apoptotic cells per 1 mm^2 seminiferous tubules areas are expressed as the means ± S.E.M. ($n=12$). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (***$P<0.001$ versus vehicle or intact; $^{a}P<0.001$ versus DBP; $^{b}P<0.001$ versus EB).

(cAMP and cGMP) and calcium, in a variety of cell types (Losel *et al.* 2003, Levin 2005, Hammes & Levin 2007). Membrane-associated ERs have been shown to prevent chemotherapy or radiation-induced apoptosis in MCF-7 cell (Razandi *et al.* 2000). Since the reduction in spermatogenic cell apoptosis by pretreatment with ICI was seen after 6 h of DBP or EB treatment in the present study, it is possible that the spermatogenic cell apoptosis induced by estrogenic compounds is at least partly mediated by this rapid nongenomic ERs signaling pathway.

DBP-induced spermatogenic cell apoptosis is independent of testicular steroidogenesis

The number of apoptotic cells was drastically decreased in the rats treated with DBP+ICI or EB+ICI compared with the number in the rats treated with DBP or EB alone. Since ICI can bind to both ERα and ERβ (Kuiper *et al.* 1998, Howell *et al.* 2000), the effects of DBP as well as EB on testes may be mediated by ERs that expressed in hypothalamus–pituitary, or possibly in testis. One of the novel findings of our study is that ICI can abolish the DBP- as well as the EB-induced spermatogenic cell apoptosis, but does not rescue the decreased ITT level and testicular steroidogenic enzyme mRNA expression (Fig. 6). Similarly, decreased level of serum FSH induced by DBP and EB was not restored by pretreatment with ICI (Fig. 7), indicating that ICI had no biological effect on hypothalamic level. The increased rate of spermatogenic cell apoptosis induced by DBP may not be due to the reduction in testicular testosterone level. In some cases, a lowered level of ITT could maintain spermatogenesis. For example, Zirkin *et al.* (1989) demonstrated that an 80% reduction in the ITT level from the control values was sufficient for the maintenance of quantitatively complete spermatogenesis. They have concluded that there is far more testosterone present within the testis of intact rats than is required for the maintenance of normal spermatogenesis. Therefore, no induction of apoptosis was detected in the DBP+ICI-treated animals in which the number of apoptotic spermatogenic cells was almost the same as that in the control animals, although the ITT level was still much lower (Fig. 6). Therefore, it is suggested that DBP-induced spermatogenic cell apoptosis is not due to ITT reduction, but may be mediated by ERs expressed in testes. The discrepant finding that ICI inhibited spermatogenic cell apoptosis but did not rescue the testicular steroidogenesis and serum FSH level reduced by DBP or EB can be explained that ICI does not cross the brain barrier and fails to block uptake of [^{3}H]-estradiol into the nuclei of hypothalamic cells (Wade *et al.* 1993, Howell *et al.* 2000). The current study that ICI abolished the DBP- or EB-induced apoptosis but could not restore testicular steroidogenesis and serum FSH level is well correlated with Wade *et al.* (1993), suggesting that it cannot penetrate across the blood–brain barrier and fails to block ERs in the hypothalamus.

In the present study, we demonstrated for the first time that estrogenic compound-induced spermatogenic cell apoptosis is not associated with testicular steroidogenesis and that ERs are directly involved in spermatogenic cell apoptosis. Indeed, ERα and ERβ are both present in rat spermatocytes and round spermatids

(Saunders *et al.* 1998, Pelletier *et al.* 2000). Moreover, using an *in vitro* model with isolated spermatogenic cells, it has been reported that estrogen directly induces spermatogenic cell apoptosis by cytochrome *c* release from mitochondria and FasL up-regulation (Mishra & Shaha 2005). However, this apoptosis was inhibited by tamoxifen, an ER antagonist, indicating that an estrogen-induced change occurs through hormone receptor interaction in spermatogenic cells. Therefore, the present observations essentially suggest the possibility that the ERs present in testes, probably in spermatogenic cells, have a role in inducing spermatogenic cell apoptosis when binding to exogenous/endogenous estrogenic compounds. However, a direct action of estrogen or estrogenic compounds on spermatogenic cell apoptosis via ERs has to be clarified in the future.

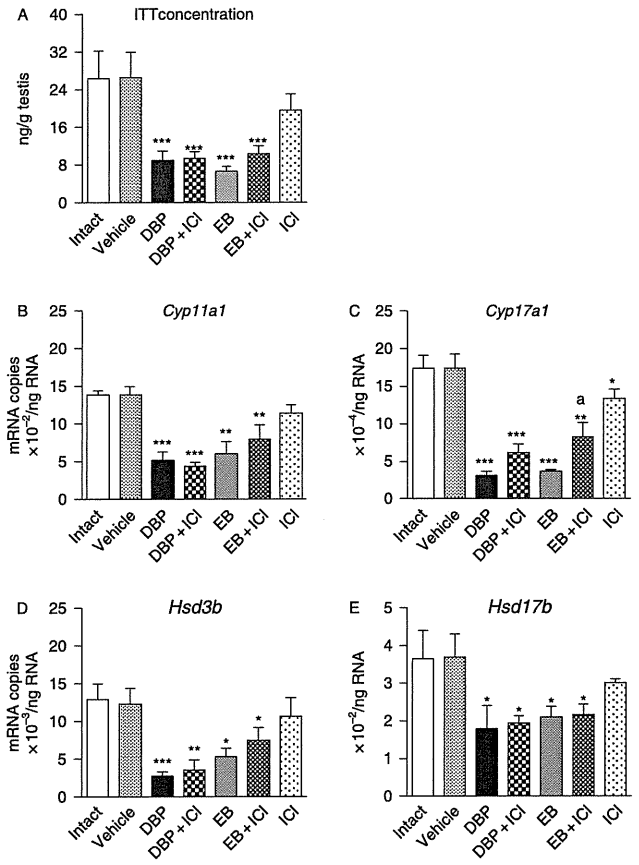

Figure 6 Comparison of ITT concentration and testicular steroidogenic enzyme gene expressions in the different treated groups. Intratesticular testosterone (ITT) level (A) and testicular mRNA expressions for *Cyp11a1* (B), *Cyp17a1* (C), *Hsd3b* (D), and *Hsd17b* (E). ITT level was measured by testosterone EIA assay, and mRNA expressions of testicular steroidogenic enzymes were analyzed by real-time RT-PCR. Results are represented as the means ± S.E.M. ($n=12$ for ITT and $n=9$ for testicular steroidogenic enzymes mRNA expressions). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*$P<0.05$; **$P<0.01$, ***$P<0.001$ versus vehicle or intact; $^{a}P<0.05$ versus EB).

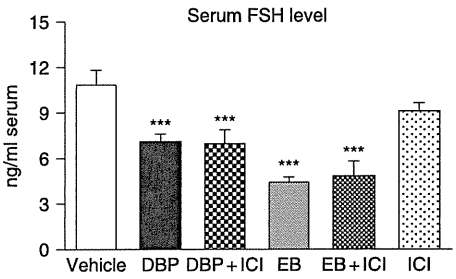

Figure 7 Serum FSH levels in the above inhibition study (Fig. 5) were measured by FSH ELISA assay. Results are represented as the means ± S.E.M. ($n=9$–12). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (***$P<0.001$ versus vehicle).

In conclusion, this is the first report of estrogenic effects of DBP *in vivo* and essentially illustrates the possible role that ERs in testes play a role in spermatogenic cell apoptosis in response to environmental estrogenic compounds. We also demonstrated that testicular steroidogenesis was not associated with spermatogenic cell apoptosis induced by estrogenic compounds. Moreover, these data will be important for understanding the role of endogenous estrogen, which might induce spermatogenic cell apoptosis via ERs for the proper maintenance of normal testicular homeostasis.

Materials and Methods

Chemicals

DBP (purity >99.8%) and EB (purity >99.0) were purchased from Sigma–Aldrich. Antiestrogen ICI 182,780 was from Tocris Cookson, Ltd (Bristol, UK). Proteinase K and 3,3′-diaminobenzidine tetrahydrochloride were from TaKaRa (Otsu, Japan). Neutral buffer formalin and propylene oxide were from Wako (Osaka, Japan). Osmium tetroxide (OsO_4) and Araldite M were from Nisshin EM Co., Ltd (Tokyo, Japan). Testosterone EIA Kit was obtained from Cayman Chemical Company (Ann Arbor, MI, USA) and rat FSH ELISA kit from ALPCO Diagnostics (Windham, NH, USA). RNeasy Mini Kit was from Qiagen. TUNEL Kit, PrimeScript RT reagent Kit (Perfect Real Time), and Ex Taq polymerase with 10× Ex Taq buffer, SYBR Premix Ex Taq (Perfect Real Time) were purchased from TaKaRa.

Animals and treatments

Male Sprague–Dawley rats (3-week-old) were purchased from Charles River Laboratories Japan (Tokyo, Japan). The rats were housed five per one plastic cage, maintained on a 12 h light:12 h darkness cycle at constant temperature (22 ± 1 °C) and humidity (45–70%), and provided water and rodent pellets (Oriental Yeast, Tokyo, Japan) *ad libitum*. Animals were maintained and handled humanely in accordance with the guidelines of the animal experiments of the Institutional Animal Care and Use Committee (IACUC) of the University of Tokyo, Tokyo, Japan.

In the first experiment, 3-week-old male rats ($n=6$) were given once daily by oral gavage for 7 days at the dose of 250, 500, or 1000 mg/kg DBP in mixture of 5% ethanol and 95% corn oil or vehicle (5% ethanol and 95% corn oil). Rats were killed using diethyl ether on the day after completion of the treatment schedule, and testes were collected, weighed, and subjected to histopathology. In the second experiment, 500 mg/kg DBP as a representative dose was given to rats ($n=8$) for 7 days in the same way as used in the first experiment. Testes were collected on the day after completion of the treatment schedule for ultrastructural observations of Leydig cells by transmission electron microscopy and analysis of testicular steroidogenesis using a Testosterone EIA Kit and real-time RT-PCR. In the third experiment, an acute DBP exposure study, rats ($n=8$) were given a single exposure of DBP (500 mg/kg) or vehicle by oral gavage and killed at 3, 6, or 24 h after administration. Then, testes and sera were collected. In the last experiment, an inhibition study with pure antiestrogen, rats ($n=12$) were i.p. administered with 3 mg/kg ICI (5% ethanol and 95% corn oil) 4 h prior to administration of DBP or EB.

The chosen dose of DBP (500 mg/kg) was based on previous reports that adverse effects on male reproductive development occur between 100 and 500 mg DBP/kg per day without systemic toxicity (Mylchreest *et al.* 2002). And the dosage of ICI was 3 mg/kg based on the previous study in which 1.5 mg/kg dosage was found to be effective estrogen antagonism (Sibonga *et al.* 1998).

Histopathology

For histopathological observations with hematoxylin and eosin or TUNEL staining, testes were immersed in 10% neutrally buffered formalin for 48 h at room temperature. Then, the samples were washed in 0.1 M PBS for 3 h, dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. The paraffin blocks were cut at 4 μm thickness. For transmission electron microscopy, rats were perfused with 5% glutaraldehyde in 0.1 M phosphate buffer, and then testes were immersed in the same fixative at 4 °C for 3 h and postfixed in 1% OsO_4 at 4 °C for 2 h. The samples were then dehydrated in ethanol, infiltrated in propylene oxide, and embedded in Araldite M. Semi-thin sections were cut at 1 μm thickness, stained with 1% toluidine blue, and observed by light microscopy. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a JEM-1010 transmission electron microscope at 80 kV (JEOL, Ltd, Tokyo, Japan).

TUNEL assay

In order to quantitatively assess the incidence of apoptotic spermatogenic cells after treatment, *in situ* TUNEL was performed by using an Apoptotic Detection Kit according to the manufacturer's instructions. Briefly, the tissue sections were deparaffinized and digested with 10 μg/ml proteinase K at 37 °C for 15 min. After being washed three to five times with 0.01 M PBS (pH 7.4), they were treated with terminal deoxynucleotidyl transferase (TdT) enzyme and labeling safe buffer, which were included in the kit. The TdT reaction was conducted at 37 °C for 90 min. After further washing three to five times with PBS, they were incubated with HRP goat anti-biotin at 37 °C for 30 min. The localization of HRP sites was determined by the application of diaminobenzidine. The sections were then counterstained with methyl green and mounted. Images of seminiferous tubules were obtained by using an OLYMPUS (BX50) light microscope connected to a digital camera (OLYMPUS, DP20, Tokyo, Japan). Under the microscope at 200× magnification, three fields in each section were randomly selected. The area of seminiferous tubules in all fields was measured by a computer-assisted system using Scion Image software (Scion Co., Frederick, MD, USA). Then, TUNEL-positive (brown-stained) spermatogenic cells in all selected area were counted. The number of TUNEL-positive cells per 1 mm^2 seminiferous tubules was calculated by dividing the total TUNEL-positive cell numbers by total seminiferous tubules area of each field and calculated accordingly in all of the fields. Data were obtained from 8–12 rats in each group and were given as mean ± S.E.M.

Hormone assay

The concentration of ITT was determined by using a Testosterone EIA Kit as previously described (Ohsako *et al.* 2003). To measure the concentration of ITT, the frozen testis was thawed and homogenized in PBS with a Polytron homogenizer (Kinematica, Luzern, Switzerland), and the testicular homogenate was extracted twice with an appropriate volume of diethyl ether. ITT level was measured according to the manufacturer's protocol. Serum LH concentration was analyzed by double antibody RIA, using materials supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The reference standard for the LH assay was NIDDK-rLH-rp-3. The intra- and inter-assay coefficients of variation (CV) for the LH assays, which were calculated from five to seven replicated determinations for the

Table 1 Primers used for real-time quantitative RT-PCR.

	Primer sequences			
Gene	Forward (genomic position)	Reverse (genomic position)	**Product size** (bp)	**Gene ID**
Cyp11a1	TGAGATCCCTTCCCCTGGTG (exon 1)	TCGCTGCGTCCTTAGGGTC (exon 2)	179	J05156
Cyp17a1	GGTCCCATCTATTCTCTTCGC (exon 1)	CTGAACACCAACTTCCGGT (exon 2)	209	M22204
Hsd3b	CTCTGGACAAAGTCTTCAGACCAGA (exon 2)	GCCTGGGTAGGACATGTGAGAC (exon 3)	183	M38178
Hsd17b	GGTTCTCCCGGTACCTTTTT (exon 1)	GTCCGGCTGATAAGTACAACA (exon 3)	166	AF035156
Ppia	GGTCCTGGCATCTTGTCCATC (exon 4)	TTCCACAATGCTCATGCCTT (exon 5)	141	M19533

pool of rat serum containing 3.0 ng/ml of LH, were 6.8 and 7.1% respectively. The serum FSH levels were determined using a rat-specific FSH ELISA kit, 29-AER004, from ALPCO Diagnostics. The manufacturer has validated this kit for the measurement of FSH in rat serum samples. The FSH assays were carried out according to the manufacturer's protocol. The sensitivity of the assay was 0.2 ng/ml. The kit was highly specific for rat FSH, with <0.1% cross-reactivity to rat GH, LH, TSH, and PRL. The intra- and inter- assay CV were 4.73 and 8.47% respectively.

Real-time RT-PCR

Total RNAs were extracted from testes by using an RNeasy Mini Kit (Qiagen). Four micrograms of total RNA samples were reverse-transcribed by using a PrimeScript RT reagent Kit (Perfect Real Time) according to the standard protocol of the supplier. Real-time PCR was performed by using SYBR Premix Ex Taq and a Light Cycler rapid thermal cycler system (Roche Molecular Systems). Table 1 shows the primer sequences, PCR product sizes, and GenBank accession numbers for all genes examined in this study – i.e. the genes encoding cytochrome P450 side chain cleavage (*P450scc, Cyp11a1*), cytochrome P450 17α/C_{17-20} lyase (*P450c17, Cyp17a1*), *Hsd3b, Hsd17b,* and cyclophilin-A (*Cp, Ppia*). The denaturation step was performed at 95 °C for 15 min, and the PCR was carried out in separate glass capillary tubes over 40 cycles (95 °C for 15 s, 60 °C for 20 s, and 72 °C for 10 s). Detection of fluorescent products was carried out at the end of the 72 °C extension period. The copy numbers of target mRNA molecules in each total RNA sample were determined by the original methods as described previously (Sakata *et al.* 2007). The mRNA levels were expressed as copy number per ng total RNA.

Statistical analysis

Statistical analysis was performed using StatView software (SAS Institute Inc., Cary, NC, USA). All results are represented as the means±S.E.M. For the comparison of testis weight, apoptotic spermatogenic cell index, serum LH, serum FSH, testicular testosterone levels, and steroidogenic enzyme gene expression levels in the dose response, time-course and inhibition studies, one-way ANOVA were carried out followed by Fisher's PLSD as a *post hoc* test. Two-tailed Student's *t*-test was used to compare the mean values of the ITT and testicular steroidogenic enzyme gene expression obtained in the repeated DBP exposure study. Differences were considered to be statistically significant when the *P* value was <0.05. Significant differences between each group are indicated in the figures where detected.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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When does the sex ratio of offspring of the paternal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure decrease: In the spermatozoa stage or at fertilization?

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ABSTRACT

Recent animal experiments confirmed that paternal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure decreases the sex ratio of offspring at birth without altering litter size. However, the timing of this decrease remained unclear. Male mice were administered TCDD at 7–12 weeks of age and mated with non-treated females. The Y-bearing/X-bearing sperm ratio was examined by real-time PCR and FISH methods, and the sex ratio of the 2-cell embryos collected from non-treated females that had been mated with TCDD-exposed males were investigated by nested PCR. The Y-bearing/X-bearing sperm ratio was not significantly decreased in the TCDD group. However, the sex ratio of the 2-cell embryos of the TCDD group was significantly lower than that of the control group. These results may have resulted from a decrease in fertility of Y-bearing sperm. Thus, the results of this study suggested that the sex ratio of the offspring was decreased at fertilization and not during the spermatozoa stage.

1. Introduction

In recent years the public has become more aware that exposure of males to certain environmental or occupational agents affects their offspring. Occupational exposure in various industries has led to increased incidences of miscarriage [1] and various birth defects [2]. It has also been shown that paternal cranial irradiation leads to epigenetic alterations in offspring [3]. Prominent among these reports are those by Mocarelli et al. [4,5] showing that paternal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in Seveso, Italy, led to a decrease in male offspring.

In 1976, TCDD was released in an explosion at a chemical plant near Seveso, Italy, resulting in the highest concentrations of TCDD ever recorded in humans. Subsequent data linked a decrease in male births in Seveso to the increased TCDD concentration in fathers, and the altered sex ratio (the proportion of male offspring) was especially pronounced in the children of fathers exposed to TCDD before age 19 [4,5]. In addition to the Seveso incident, Ryan et al. [6], in a study conducted in Ufa, Russia, suggested that human exposure to high levels of dioxin is associated with the birth of more girls only in cases of paternal exposure. In our previous study, we exposed young male mice to two concentrations of TCDD (TCDD2/0.4 group and TCDD2000/400 group; an initial loading dose of 2 or 2000 ng TCDD/kg, followed by a weekly maintenance dose of 0.4 or 400 ng TCDD/kg) to re-create the Seveso incident and evaluated the sex ratio of their offspring at birth [7]. The reason why we used young male mice (7 weeks at the start of administration) in that study was that the sex ratio of offspring of males exposed to TCDD during adolescence showed a greater decrease than that of males who were older than that when the incident occurred [4,5]. The results from the previous study revealed that paternal TCDD administration produced a dose-dependent reduction in the sex ratio of offspring (F1) and a significantly lower proportion of male offspring in the high-dose (TCDD2000/400) group. In addition, the induction intensity of CYP1A1 in the liver varied among individuals in the TCDD group, and the dimensions of the CYP1A1 immunoreactive area were correlated with the sex ratio of the offspring. This means that the high sensitivity subgroup of male parents to TCDD was strongly related to the decrease in male offspring. We also reported that TCDD exposure does not influence

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litter size; the number of male offspring decreases by TCDD exposure, while the number of female offspring increases. From these data, we presumed that paternal TCDD exposure decreased the sex ratio of offspring and altered it before implantation occurred. However, the mechanisms underlying the reduction in male offspring and the timing of this change have remained unclear.

The purpose of this study was to investigate when the sex ratio of the offspring decreases. We examined the Y-bearing/X-bearing sperm ratio as well as the sex ratio of 2-cell embryos by exposure to the same dose (an initial loading dose of 2000 ng TCDD/kg, followed by a weekly maintenance dose of 400 ng TCDD/kg) as used in our previous study [7], because this dose group showed a significant decrease in the sex ratio of offspring at birth. The effects of TCDD exposure to sperm concentration and motility were also examined.

2. Materials and methods

2.1. Chemicals

TCDD was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sesame oil, used for dissolving TCDD and for vehicle treatment as a control, was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.2. Animals and treatments

Male and female ICR mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and bred at Kobe University (Kobe, Japan). They were maintained under controlled conditions of temperature (23 ± 2 °C) and humidity (50 ± 10%) on a 12 h light, 12 h dark cycle. The animals were given an MR-A1 laboratory diet (Nosan Corporation, Yokohama, Japan) and filtered water *ad libitum* throughout the experiments. This study was approved by the Institutional Animal Care and Use Committee (Permission number: 19-5-46) and carried out according to the Kobe University Animal Experimentation Regulations.

We used 7-week-old male mice at the start of administration to re-create the Seveso incident. These male mice were divided into a control group ($n = 59$) and a TCDD group ($n = 49$), and were administered TCDD orally by gastric sonde with an initial loading dose of 2000 ng TCDD/kg body weight or an equivalent volume of sesame oil (vehicle) as a control, followed by a weekly maintenance dose of 400 ng TCDD/kg body weight to maintain the body TCDD burden as constantly as possible or an equivalent volume of sesame oil until the mice reached 12 weeks of age. We also used 6-week-old female mice ($n = 62$) for mating with males.

2.3. Epididymal sperm concentration and motility analysis

Mouse spermatozoa were collected from TCDD-treated mice and control mice for examination of sperm concentration and sperm motility. The mice were sacrificed by cervical dislocation under anesthesia using ether, and the caudal epididymides were then dissected out. The epididymis was cut at one or two points with a scissors, and white sperm pellets were released into a TYH-HEPES medium droplet that was covered with mineral oil (Nacalai Tesque Inc., Kyoto, Japan) on a 37.5 °C heater plate. The TYH-HEPES medium was composed of 119.37 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 1.00 mM sodium pyruvate, 5.56 mM glucose, 25.07 mM HEPES, 0.05 g/l streptomycin sulfate, 100 U/ml potassium penicillin G, 0.1% polyvinylalcohol (Sigma–Aldrich Co., St. Louis, MO, USA) and 5 mg/l phenol red. The recovered spermatozoa were diffused for 5 min in the droplet. The sperm concentration was measured using a hemocytometer chamber. For sperm motility analysis, a 2 μl drop of sperm suspension was put on a 1-mm-deep stage of a glass plate to assess sperm motility (Fujihira Industry Co., Ltd., Tokyo, Japan), covered with the coverslip and then placed on the heated plate (37.5 °C) under a bright-field microscope (EX41; Olympus Co., Tokyo, Japan). Sperm motility was recorded with a CCD camera (CS230B; Olympus Co.) and a DVD recorder (DVR-7000; Pioneer Co., Tokyo, Japan). The motility patterns of more than 100 spermatozoa (except large sperm clumps) were randomly recorded with the CCD camera and DVD recorder in each sample. The recorded movies were played in the slowest mode. At least 10 microscopic fields were observed for each sample, and the percentage of motile sperm was determined.

2.4. Sperm DNA isolation

Mouse spermatozoa for DNA isolation were collected from TCDD-treated and control mice by pricking the caudal epididymides with needles, and this manipulation was done in a TYH-HEPES medium droplet that was covered with mineral oil under a stereomicroscope so as not to contaminate the other male tissues. The sperm pellets were resuspended with a 1–1.5 volume of lysis buffer containing 0.5 M EDTA, 2-mercaptoethanol (Sigma–Aldrich Co.) and 10 mg/ml proteinase K (Takara Bio Inc., Shiga, Japan), and then incubated in a shaking water bath at 55 °C overnight. The 2-mercaptoethanol was used to obtain high-quality spermatozoa DNA yields for real-time PCR, because the spermatozoa DNA is tightly packed into protamines. Sperm DNA extraction was performed using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA) following the manufacturer's instructions.

2.5. Real-time PCR and calculation of Y-bearing/X-bearing sperm ratio

To gain an accurate prediction of the X and Y chromosome content in the sperm DNA samples, quantitative real-time polymerase chain reaction (PCR) analysis was performed using a LightCycler (Roche Co., Basel, Switzerland). We selected the *Sry* primer pair (forward: 5′-ATGGAGGGCCATGTCAAGCG-3′ and reverse: 5′-GGGTATTTCTCTCTGTGTAGGATCTTCAA-3′) as Y-bearing sperm-specific primers and the *AR* primer pair (forward: 5′-ATGGAGGTGCAGTTAGGGCT-3′ and reverse: 5′-TCCTCAGTGTGCTGCTGCC-3′) as X-bearing sperm-specific primers. To calculate Sry and AR gene copy numbers in the isolated genomic DNA, the basic protocol for real-time PCR was briefly modified [8]. The Sry and AR gene PCR products were used for calibration by calculating the molecular weight and making stock dilutions from 2×10^8 to 2×10^3 copies/μl. Aliquots (2 μl) of DNAs or standard DNA solution were amplified with a master mixture (SYBR Premix Ex Taq (Perfect Real Time), TaKaRa Bio Inc.) containing the Sry- or AR-specific primers described above in a final volume of 20 μl. Fluorescent products were detected at the end of the extension period. The specificity of the amplified PCR products was confirmed by melting curve analysis. Unknown concentrations of samples were extrapolated by a comparison with standards amplified under the same conditions using Lightcycler software. The Y-bearing/X-bearing sperm ratio [DNA concentration of Sry/DNA concentration of AR] of each animal was calculated.

2.6. Preparation of epididymal sperm smears for FISH

Mouse spermatozoa for FISH analyses were collected from TCDD-treated and control mice. Mice were killed by cervical dislocation, and then the caudal epididymides were dissected out. The epididymis was cut at 1 or 2 points with a scissors, and the white sperm pellet was placed into 0.1 ml of 2.2% sodium citrate at 32 °C for 5 min to allow the sperm to swim out into the solution. The sperm suspension was centrifuged at 1500 rpm for 5 min. The sperm pellet was resuspended into 0.1 ml of 0.075 M KCl for 45 min at 37 °C. Sperm suspension (5 μl) was pipetted onto a dry glass slide that had been precleaned by soaking in 100% ethanol for at least 24 h. The smears were allowed to air-dry overnight and then stored at −20 °C until used.

2.7. FISH in spermatozoa and calculation of Y-bearing sperm ratio

Smears of mice were each fixed in 3:1 methanol: acetic acid and air dried before pretreatment commenced. To prepare the smears for FISH, spermatozoa were decondensed by incubating the slides for 30 min in 0.2 ml of 10 mM dithiothreitol (Sigma–Aldrich Co.) on ice. Slides were briefly rinsed in D.W. and allowed to dry completely at room temperature before they were used for hybridization. The probes specific for mouse Y chromosome (Cy3-labeled) were obtained from Cambio (Cambridge, UK). The probe mixture, which included labeled probes and hybridization buffer, was denatured at 72 °C for 10 min. The sperm smears were denatured at 78 °C for 6 min in 70% formamide/2xSSC and then dehydrated in an alcohol series (70%, 85% and 100%, 2 min each). The probe mixture was applied to the sperm smears and incubated at 37 °C overnight. After hybridization, the slides were washed six times: three times in washing solution (50% formamide/2xSSC), twice in 2xSSC, and once in PN buffer for 5 min each at 37 °C. The nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) (Cambio Ltd.). Coverslips were applied and sealed with clear nail polish. Slides were then viewed.

The slides were examined with a Leica FW4000 fluorescence microscope (Leica Co., Wetzlar, Germany) (magnification: 400×) equipped with single and double band-pass filters to detect DAPI and Cy3. The investigation was performed blindly, and at least 1000 cells per slide were scored as much as possible by assessing randomly selected visual fields. Sperm nuclei were scored only when they were morphologically preserved, not clumped or overlapping, when they showed well-defined outlines and when the sperm heads were not decondensed to more than twice the size of normal non-decondensed spermatozoa. In every sample, the proportion of sperm presenting with a clear signal was ≥95%.

The Y-bearing sperm ratio [Y-chromosome-bearing spermatozoa/DAPI-positive spermatozoa × 100] of each animal was calculated.

2.8. Embryo collection for nested PCR

Six-week-old female mice were superovulated by intraperitoneal injections of 5 IU eCG (Teikoku Hormone Medical Co., Ltd., Tokyo, Japan) followed 48 h later by intraperitoneal injections of 5 IU of hCG (Teikoku Hormone Medical Co., Ltd.). One week after the last exposure of TCDD to male mice, each superovulated female mouse that had not been exposed to TCDD was paired with a male, with one pair per cage. After confirmation of vaginal plug in each female, embryos were collected 44 h after hCG injection from the oviducts of the females at the 2-cell stage in 37 °C FHM medium, which was composed of 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 4 mM $NaHCO_3$, 0.2 mM glucose, 0.2 mM glutamine, 0.2 mM Na-pyruvate, 10 mM Na-lactate, 1.71 mM $CaCl_2 \cdot 2H_2O$, 0.01 mM EDTA·4Na, 20 mM HEPES, 80 mg/l kanamycin and 1 g/l BSA.

The embryos were transferred to 80 μl drops of KSOM medium, which was composed of 94.97 mM NaCl, 2.55 mM KCl, 0.37 mM KH_2PO_4, 0.23 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 0.22 mM glucose, 1.0 mM glutamine, 0.2 mM Na-pyruvate, 10 mM Na-lactate, 1.7 mM $CaCl_2 \cdot H_2O$, 80 mg/l kanamycin, 3 g/l BSA, 0.1 mM EDTA–2Na under mineral oil and separated by gentle pipetting of the groups of 2-cell embryos using a micropipette with an internal diameter of 70–100 μm. Each 2-cell embryo was put into a PCR micro-test-tube containing 15 μl of 0.15 mg/ml proteinase K with the help of a dissection microscope. After the embryos were collected, the PCR tubes were heated for 60 min at 50 °C and then for 10 min at 100 °C to inactivate the proteinase K. The cells in the PCR micro-test-tubes were stored at −80 °C until PCR analysis was performed.

2.9. Oligonucleotide primers for nested PCR

The *Sry* gene was chosen as the Y chromosome-specific gene and the *IL-3* gene was chosen to detect both XX and XY embryos. For every locus, two sets of primers were used, an inner and an outer set. The primers were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). The oligonucleotide sequences from the outer primers used in the initial PCR were as follows:

Sry-outer forward 5′-TCTTAAACTCTGAAGAAGAGAC-3′
Sry-outer reverse 5′-GTCTTGCCTGTATGTGATGG-3′
IL-3-outer forward 5′-GGGACTCCAAGCTTCAATCA-3′
IL-3-outer reverse 5′-TGGAGGAGGAAGAAAAGCAA-3′
The inner primer sequences used in the second-round PCR were as follows:
Sry-inner forward 5′-TTCCAGGAGGCACAGAGATT-3′
Sry-inner reverse 5′-GTCCCACTGCAGAAGGTTGT-3′
IL-3-inner forward 5′-GGGAAGCTCCCAGTGAGTAA-3′
IL-3-inner reverse 5′-GGTTCACCCACAGCTGTCTT-3′

The sequences of Sry-outer primers were the same as those used in Kunieda et al. [9].

2.10. Nested PCR and the sex ratio of the 2-cell embryos

A multiplex PCR reaction using nested primers was performed in two rounds for simultaneous amplification of the *Sry* and *IL-3* sequences. Samples were thawed on ice, and a 15 μl first-round PCR mix containing the outer primers was added. After the first amplification, 1 μl portions of the products of the amplification reactions were dispensed respectively into each tube, and each was subjected to second-step amplification with 9 μl of a reaction mixture containing the corresponding inner primers. Both steps of the PCR were carried out using a reaction mixture consisting of a 5× PCR buffer, 2.5 mM dNTPs, 50 μM each of the oligonucleotide primers and 1.25 U/μl of Taq DNA polymerase (Promega). PCR was performed for 30 cycles, each consisting of 1 min denaturation at 94 °C followed by annealing and extension for 1 and 1.5 min each at 55 and 72 °C, respectively. The PCR products were subjected to electrophoresis in a 2.5% agarose gel containing 0.005% ethidium bromide. The male embryos showed both Sry and IL-3 signals, but the female embryos showed only the IL-3 signal.

The sex ratio of the 2-cell embryos [number of male embryos/number of (male + female) embryos × 100] of each animal was calculated.

2.11. Statistical analysis

SAS Ver 5.0 (SAS Institute, Cary, NC, USA) was used to analyze the present data. In comparing every parameter between two groups (control and TCDD groups), the equality of variances was evaluated by using the *F* test to select statistical tests. If variances were homoscedastic, we used Student's *t*-test. In contrast, if the variances of parameters were not homoscedastic, the Welch test was applied. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Epididymal sperm concentration and motility

Epididymal sperm concentrations of five males per group and sperm motility of seven males in the control group and five males in the TCDD group were examined. Figs. 1 and 2 show the effects of TCDD exposure on sperm concentration and motility. Compared with the control group, the TCDD group showed a lower level of caudal epididymal sperm concentration [Control: $4.28 \pm 0.79 \times 10^7$/ml; TCDD: $2.29 \pm 0.70 \times 10^7$/ml; $P = 0.10$], because one of the mice in the TCDD group showed a very low sperm concentration. The mean value of sperm motility in the TCDD group was lower than that of the control group, but the difference was not significant [Control: 45.39 ± 7.19%; TCDD: 42.48 ± 5.71%; $P = 0.77$].

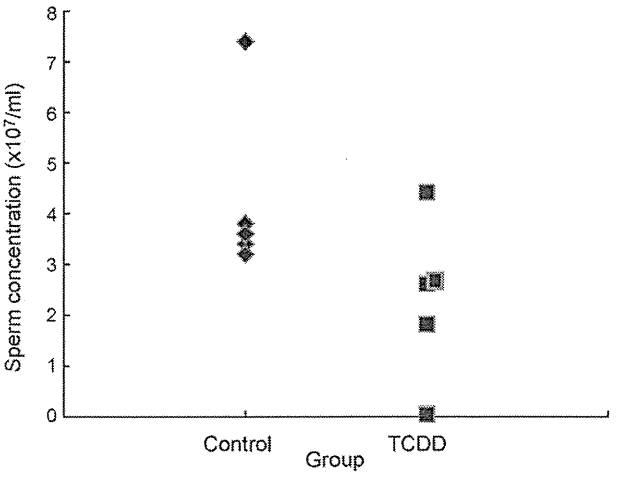

Fig. 1. Concentration of epididymal spermatozoa. The sperm concentration of the TCDD group was lower than that of the control group (Control: $4.28 \pm 0.79 \times 10^7$/ml; TCDD: $2.29 \pm 0.70 \times 10^7$/ml; $P = 0.10$). The sample size was five per group ($n = 5$).

3.2. Y-bearing/X-bearing sperm ratio

The results of quantitative PCR are shown in Fig. 3A–C. The control group consisted of 20 male mice and the TCDD group consisted of 14 male mice. Although the Y-bearing/X-bearing sperm ratio of the TCDD group was lower than that of the control group [Control: 2.68 ± 0.15, TCDD: 2.36 ± 0.04; $P = 0.060$], the difference was not significant. In addition, the TCDD group tended to have lower levels of Sry DNA concentrations [Control: 28.12 ± 1.20 ng/μl, TCDD: 25.80 ± 0.61 ng/μl ($P = 0.096$)], which is a sensitive and specific marker of the Y chromosome. This difference also did not attain statistical significance. The DNA concentrations of AR showed no differences between the control and TCDD groups [Control: 10.87 ± 0.57 ng/μl, TCDD: 10.95 ± 0.29 ng/μl, $P = 0.895$].

3.3. Y-bearing sperm ratio examined by FISH method

A total of 10,083 spermatozoa from five males per group were scored with the FISH technique (Fig. 4). As shown in Table 1, no significant differences were found between the groups. The sperm

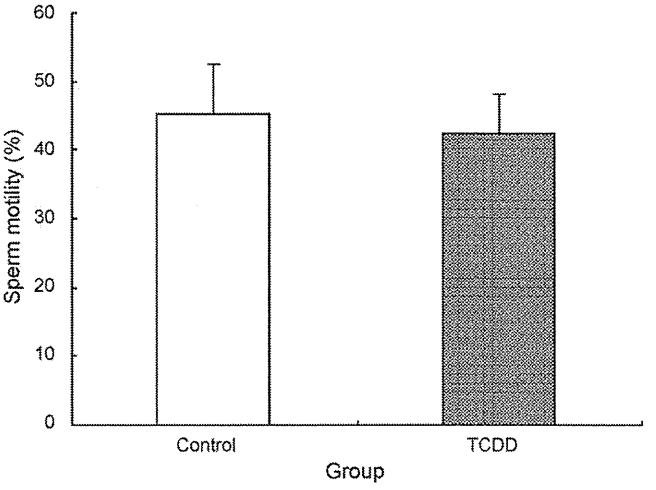

Fig. 2. Motility of epididymal spermatozoa. There were no apparent changes in the motility of the cauda epididymal spermatozoa in the TCDD group compared with the control group ($P = 0.77$). However, the mean value in the TCDD group was lower than that in the control group (Control: 45.39 ± 7.19%; TCDD: 42.48 ± 5.71%). The sample size of the control group was seven, and that of the TCDD group was five. Each histogram and bar indicate mean ± SE.

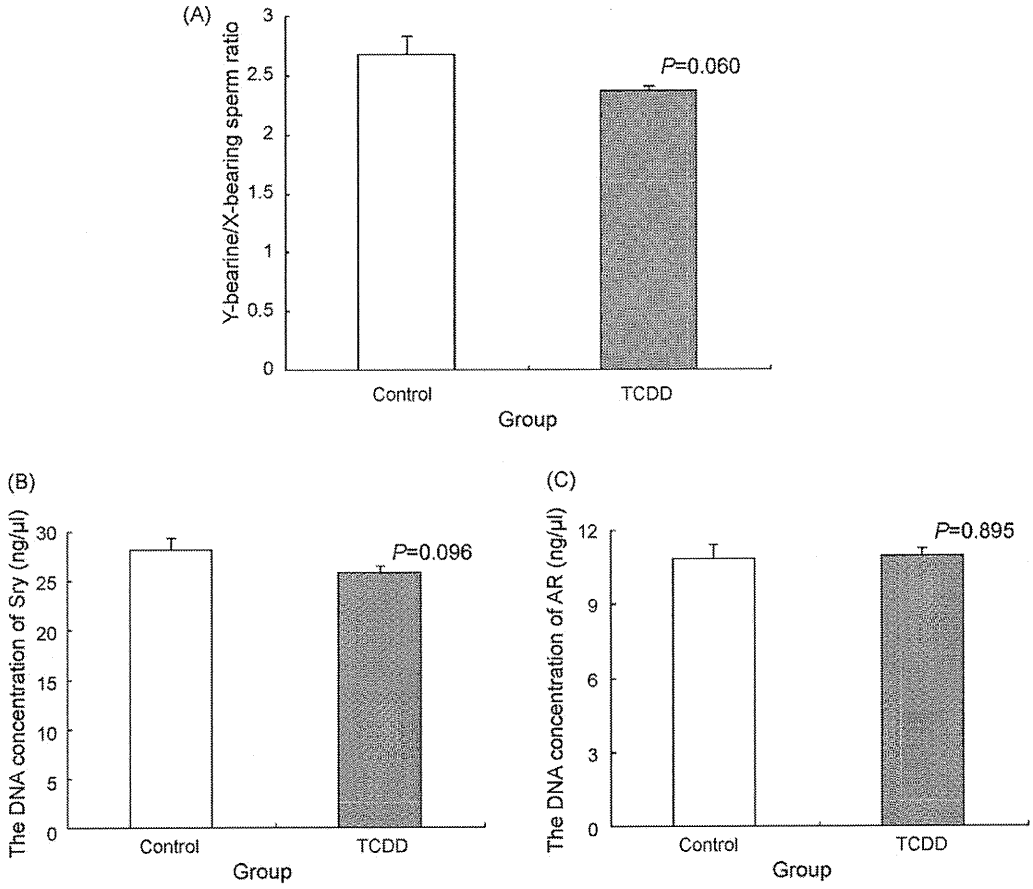

Fig. 3. (A) Y-bearing/X-bearing sperm ratio. The Y-bearing/X-bearing sperm ratio in the TCDD group did not show a significant decrease compared with the control group. However, the Y-bearing/X-bearing sperm ratio of the TCDD group was lower than that of the control group. The sample size of the control group was 20, and that of the TCDD group was 14. (B) The concentrations of the sperm DNA of Sry. The Sry primer was used to detect Y-bearing sperm. The concentrations of sperm DNA of Sry in the TCDD group were lower levels than those of the control group. (C) The concentrations of the sperm DNA of AR. The AR primer was used to detect X-bearing sperm. The concentrations of AR showed no apparent changes between the control group and the TCDD group. Each histogram and bar indicate mean ± SE.

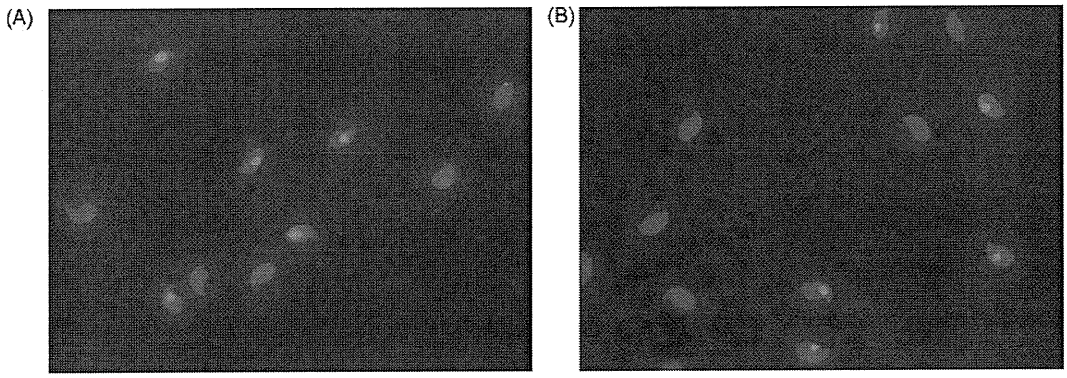

Fig. 4. Microscope images of FISH in epididymal spermatozoa. Samples were hybridized with chromosome-specific painting DNA probes for Y chromosomes (Cy3/red). The sperm nuclei were stained with DAPI and appear as blue. (A) Sperm samples of the control group yielded nearly equal ratios of X and Y chromosome-bearing cells. (B) Microscope images of FISH in epididymal spermatozoa of the TCDD group. There was no significant change in the Y-bearing sperm ratio of the TCDD group compared with the control group. The sperm nuclei were stained with DAPI.

Table 1
Y-bearing sperm ratio examined by FISH method.

Group	Control						TCDD					
Animal no.	1	2	3	4	5	Total	1	2	3	4	5	Total
Cells scored	1055	1071	1080	1086	1068	5360	1074	1079	1094	308	1168	4723
Y sperm	532	539	541	553	536	2701	539	540	549	149	586	2363
X sperm	523	532	539	533	532	2659	535	539	545	159	582	2360
Y-bearing sperm ratio (%)	50.43	50.33	50.09	50.92	50.19	50.39	50.19	50.05	50.18	48.38	50.17	50.03

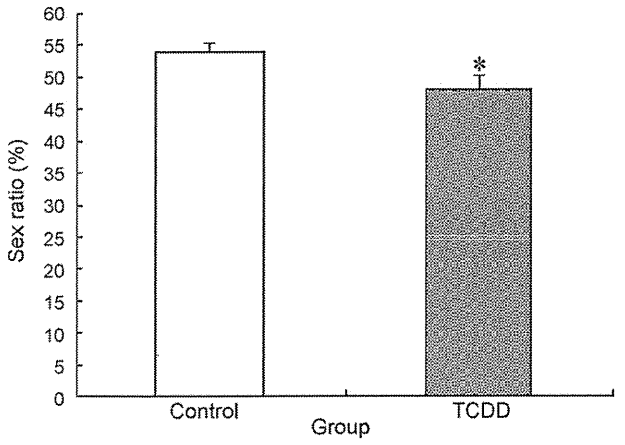

Fig. 5. Sex ratio of 2-cell embryos. A total of 614 embryos of the control group and 646 embryos of the TCDD group were examined. The proportion of XY 2-cell embryos from the TCDD-exposed sires was significantly lower than that of the control group. Each histogram and bar indicate mean ± SE. *: $P < 0.05$.

count scored with the FISH technique from one of the five mice in the TCDD group was very low, because the total number of sperm collected from the cauda epididymides of that mouse was much lower than that of any of the others.

3.4. Sex ratio of 2-cell embryos

The sex ratio of the 32 litters from the control group (614 embryos) and 30 litters from the TCDD group (646 embryos) were sexed by nested PCR using XY embryo-specific primers (Fig. 5). We found a statistically significant decrease in the sex ratio of embryos in the TCDD group compared with the control group [Control: 53.95 ± 1.54%, TCDD: 47.92 ± 2.20%, $P < 0.05$].

4. Discussion

In theory, the sex allocation ratio of many species of mammals is almost 1:1. However, in reality this is not necessarily true. There have been many reports about changes in the sex ratio of human offspring. Severe periconceptional life events have been found to reduce the sex ratio in offspring [10]. The proportion of male offspring decreases with increasing parental age, and is higher in white people than in black people [11]. The sex ratio varies with the coital rate and with the time taken to achieve conception [12]. The proportion of male offspring to female offspring decreased after the Kobe earthquake [13]. Among these reports about alterations in the sex ratio of offspring, reports that TCDD exposure altered the ratio have attracted attention. A decreased male/female sex ratio among children born to males exposed to TCDD at a relatively young age compared with unexposed males has been reported in Seveso, Italy [4,5] and Ufa, Russia [6]. Still other reports found no significant association between paternal serum TCDD levels and the sex ratio of offspring in the USA [14] and Japan [15]. In animal experiments, Ikeda et al. [16] showed that TCDD exposure to male rats *in utero* significantly decreased the number of male offspring. In contrast, Rowlands et al. [17] showed that the number of rat male offspring was not decreased by *in utero* TCDD exposure. Thus, based on these previous articles, it was unclear whether or not TCDD exposure altered the sex ratio of offspring, and no experiments re-creating the Seveso incident had been conducted. Therefore, in our previous study we exposed two different doses of TCDD only to sexually mature young male mice (F0) and examined TCDD's effect on the sex ratio of their offspring (F1) at birth [7]. The results showed that the male/female sex ratio of the offspring dose-dependently decreased in the TCDD groups, and that of the high-dose group significantly decreased. Despite changes in the sex ratio, no alteration was found in the litter size of the TCDD group compared to the control group [7]. The data suggest that direct paternal TCDD exposure, not *in utero* exposure, decreases the sex ratio of offspring, and that the sex ratio of offspring already changes before implantation signs are seen. However, the timing of the alteration of the sex ratio of offspring in TCDD-exposed male parents remained unclear, so we investigated TCDD exposure's effect on the Y-bearing/X-bearing sperm ratio and the sex ratio of 2-cell embryos.

In the present study, the Y-bearing/X-bearing sperm ratio was examined by using a modified version of Parati's method [18] of quantitative PCR. This ratio did not show a significant decrease between the control group and the TCDD group. However, the Y-bearing/X-bearing sperm ratio and DNA concentrations of Sry of the TCDD group tended to decrease, with $P = 0.06$. On the other hand, the DNA concentration of AR was not affected by TCDD exposure. The Y-bearing sperm ratio was also checked by the FISH method, which revealed no marked differences between the groups. This result supports the findings of real-time PCR.

In this study, sperm motility was not significantly decreased by TCDD administration, but the mean value of that of the TCDD group was lower than that of the control group. In addition, the sperm concentration of the TCDD group was lower than those of the control group, with $P = 0.10$. Mocarelli et al. [19] reported that exposure to TCDD in infancy significantly reduced sperm concentration and motility, whereas an opposite effect was seen with exposure during puberty in Seveso, Italy. However, in the present study, the sperm concentration and motility were not significantly decreased. Our results did not coincide with those of a report on humans [19]. The causes of difference between ours and humans are that the parameters from sperm samples collected 22 years after the accident were measured in the human study, whereas we exposed TCDD to male mice from 7 to 12 weeks of age after puberty and examined their sperm almost immediately (1 week) after exposure. In addition, our TCDD administration dose was much lower than that in the Seveso incident. Thus, the differences in when the sperm samples were collected and in the exposed dose between Mocarelli's study [19] and the present one might account for the lack of a significant decreases in sperm concentration and motility in the present study.

The sex ratio of the 2-cell embryos in the TCDD group showed a significant decrease compared with the control group. This indicates that the sex ratio of the TCDD group had already decreased at the 2-cell embryo stage.

Thus, TCDD exposure significantly reduced the sex ratio of the embryos and the sex ratio at birth without altering litter size. However, TCDD exposure did not affect the Y-bearing/X-bearing sperm ratio. These results suggest that the sex ratio of offspring was decreased at fertilization, and thus that the sex ratio of neonates was also decreased. In the case of paternal TCDD exposure, the number of eggs per litter was not affected, therefore we think this is because the fertility of Y-bearing sperm might have been affected by TCDD exposure, and thus more X-bearing spermatozoa than Y-bearing spermatozoa were fertilized. As a result, both the sex ratio of embryos and that at birth were decreased in the TCDD group with no change in litter size. This distortion of the sex ratio might be produced by epigenetic alterations induced by TCDD exposure [20,21]. However, the mechanisms of decrease in the sex ratio, especially in the fertility of Y-bearing spermatozoa, remain to be investigated.

In conclusion, this study suggests that TCDD exposure to only male mice significantly decreases the sex ratio of 2-cell embryos without altering the Y-bearing/X-bearing sperm ratio. Our previous study revealed that the sex ratio at birth was significantly decreased by TCDD exposure despite no difference in litter size [7]. That finding, together with the current results, leads us to conclude that the sex ratio of offspring was decreased at fertilization in the TCDD exposure cases. Further investigation of the

differences in fertility between Y-bearing and X-bearing spermatozoa is needed.

Conflicts of interest

The authors have no conflicts of interest that would have inappropriately influenced the work presented in this manuscript.

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