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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.

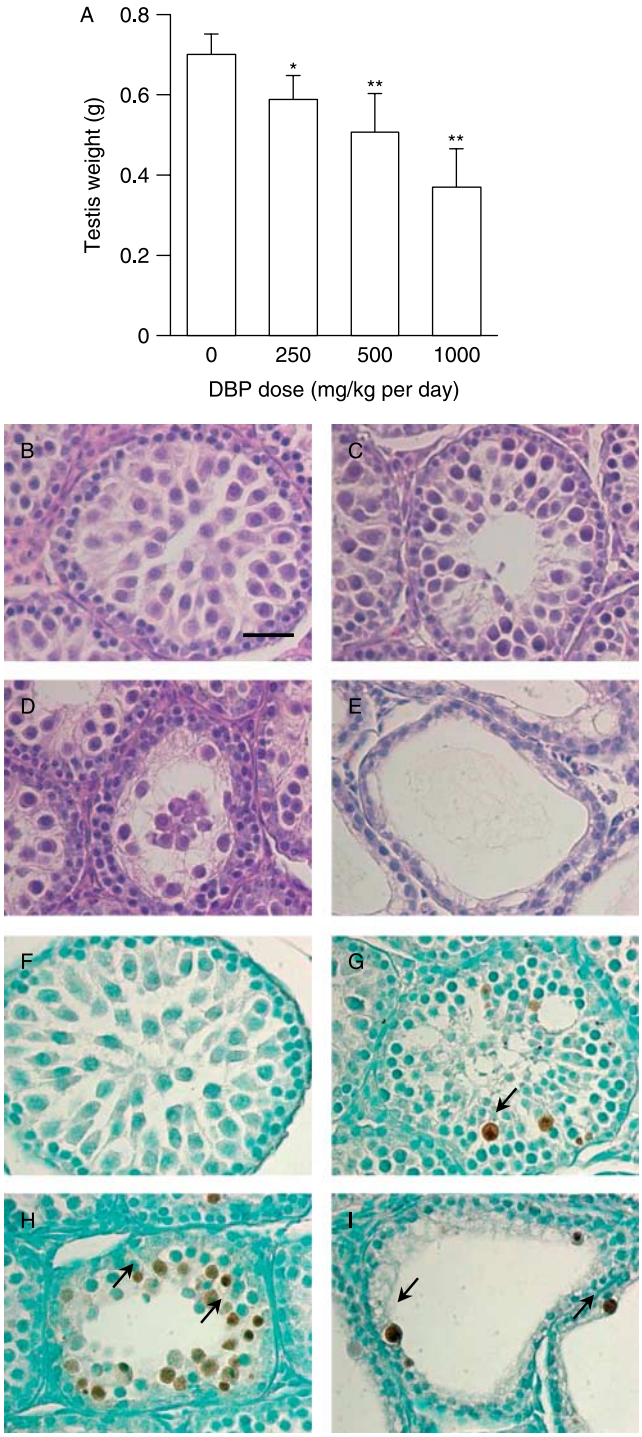

Figure 1 Effects of repeated DBP exposure on testis morphology. Testis weight after once daily administration of DBP for 7 days (A) is shown. Results are expressed as the means±S.E.M. ($n=6$). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (*$P<0.05$; **$P<0.01$ 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.

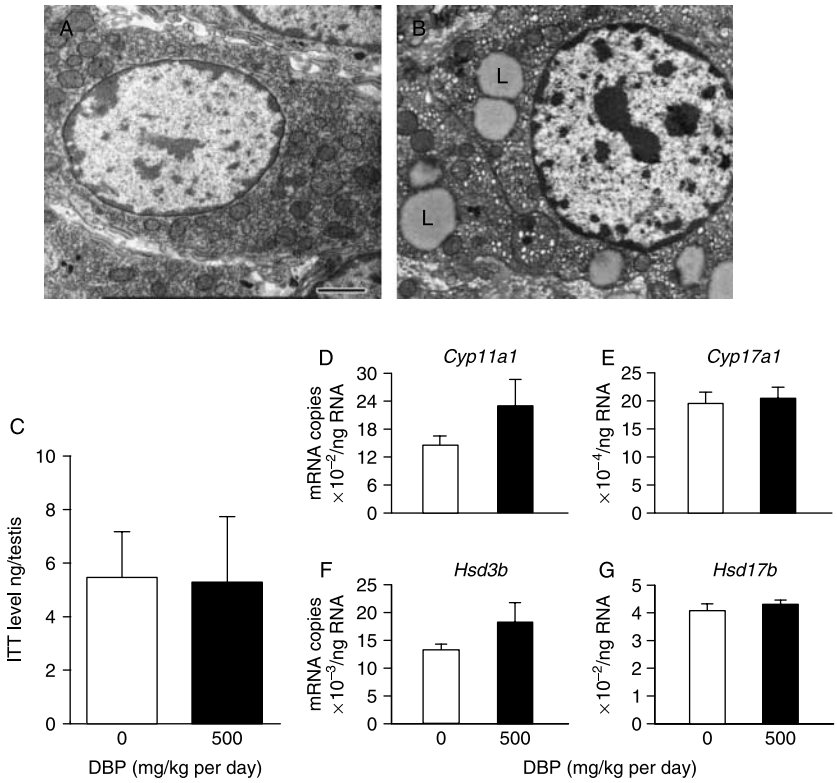

Figure 2 Effects of repeated DBP exposure on testicular steroidogenesis. Ultrastructural changes in Leydig cells of the control (A) and 500 mg/kg per day treated groups (B). L indicates lipid droplets. Scale bar, 1 μm. Intratesticular testosterone (ITT) level (C) and testicular mRNA expressions for *Cyp11a1* (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

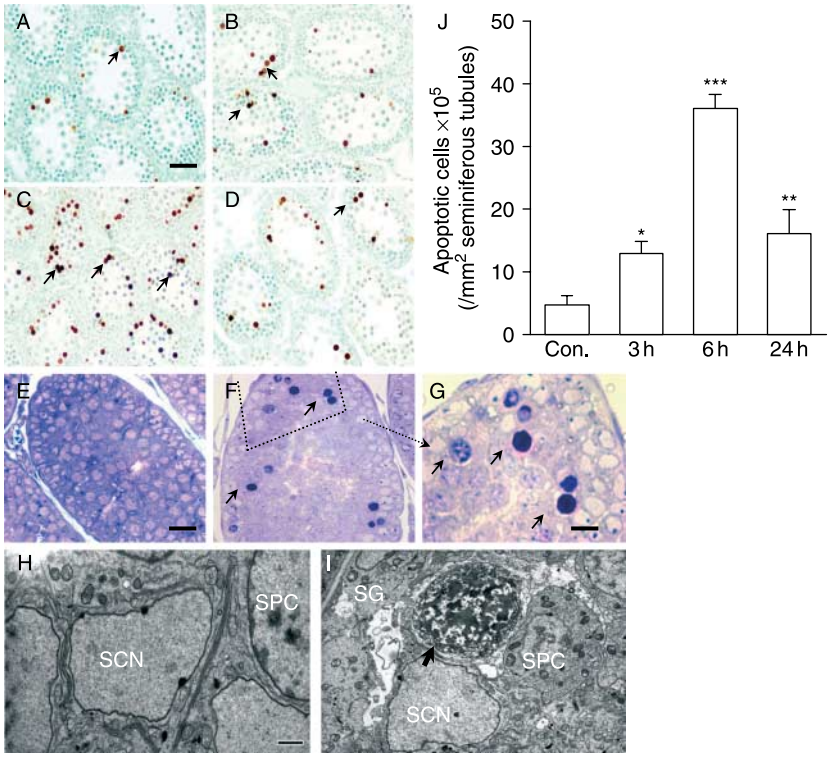

Figure 3 Spermatocytes apoptosis after a single exposure of 500 mg DBP/kg. TUNEL labeling of testes. Vehicle-treated control (A), and at 3 h (B), at 6 h (C), and at 24 h (D) after treatment. Note the maximal apoptotic spermatogenic cell number at 6 h after treatment. Arrows indicate apoptotic spermatocytes. 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

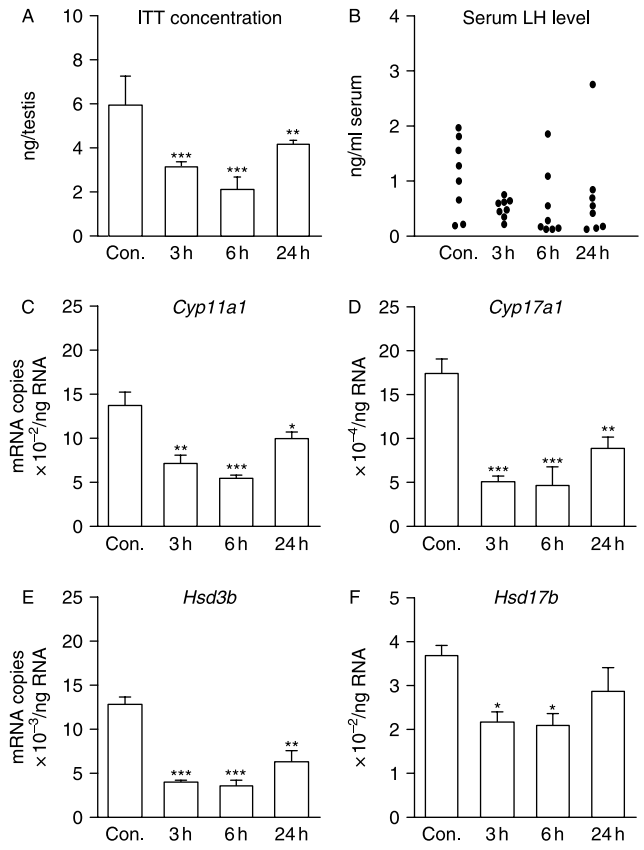

Figure 4 Testicular steroidogenesis and serum LH levels after a single exposure of 500 mg DBP/kg. Intratesticular testosterone (ITT) level (A), serum LH level (B), and testicular mRNA expressions for *Cyp11a1* (C), *Cyp17a1* (D), *Hsd3b* (E), and *Hsd17b* (F). ITT level was measured by testosterone EIA 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

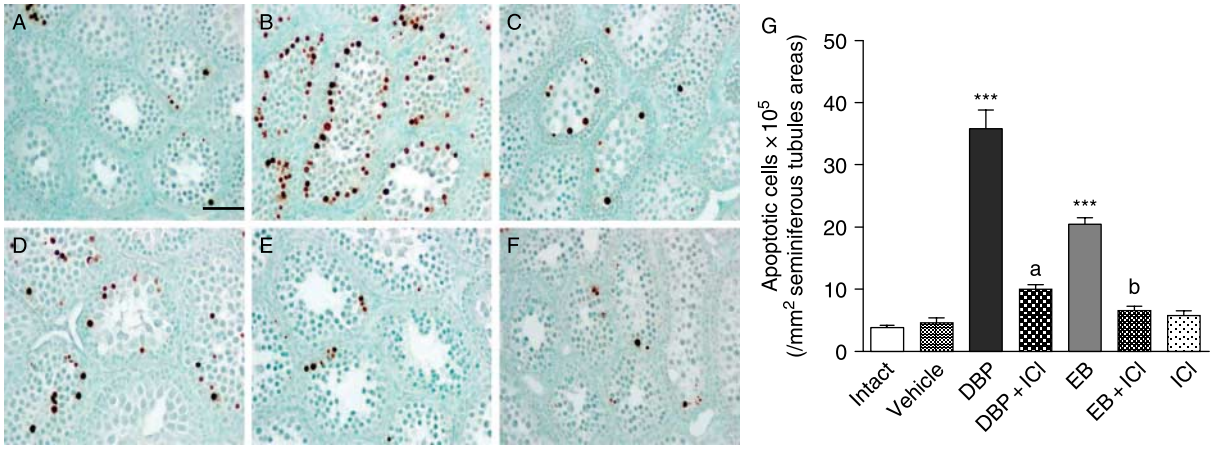

Figure 5 ICI inhibits DBP- or EB-induced spermatogenic cell apoptosis. Results are shown for the control (A), DBP alone (B), DBP+ICI (C), EB alone (D), EB+ICI (E), and ICI alone (F) groups. Rats ($n=12$) were i.p. administered with 3 mg/kg of ICI (5% ethanol and 95% corn oil) 4 h prior to administration 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.

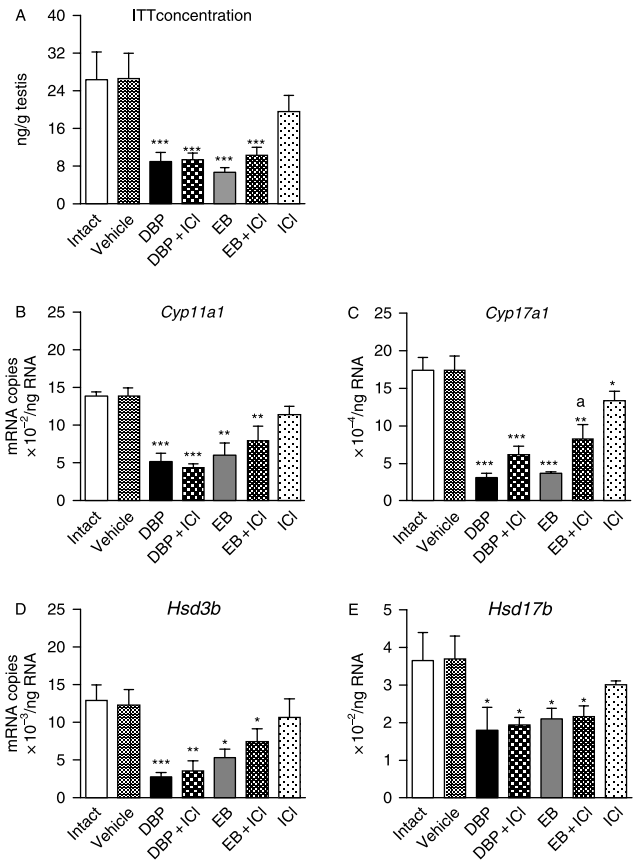

Figure 6 Comparison of ITT concentration and testicular steroidogenic enzyme gene expressions in the different treated groups. Intratesticular testosterone (ITT) level (A) and testicular mRNA expressions for *Cyp11a1* (B), *Cyp17a1* (C), *Hsd3b* (D), and *Hsd17b* (E). ITT level was measured by testosterone 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).

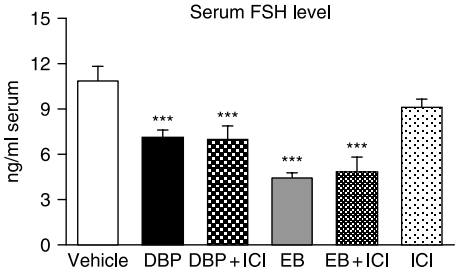

Figure 7 Serum FSH levels in the above inhibition study (Fig. 5) were measured by FSH ELISA assay. Results are represented as the means ± S.E.M. ($n=9$–12). Statistically significant differences were determined by ANOVA followed by Fisher's PLSD test (***$P<0.001$ versus vehicle).

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$\pm$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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