

Bisphenol A Inhibits Testicular Functions and Increases Luteinizing Hormone Secretion in Adult Male Rats

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Effects of a xenobiotic estrogen, bisphenol A (BPA), on reproductive functions were investigated using adult male rats. BPA was dissolved into sesame oil and injected sc every day (1 mg/rat) for 14 days. Animals were killed by decapitation after the final administration of BPA, and the trunk blood, pituitary, and testes were collected. Plasma concentrations of prolactin were dramatically increased and pituitary contents of prolactin were slightly increased in the BPA group compared to the control group. Plasma concentrations of testosterone were decreased and plasma concentrations of LH were increased in BPA-treated rats compared to control rats. Testicular contents of inhibin were decreased in BPA-treated rats compared to control rats, although plasma concentrations of inhibin were not changed after administration of BPA. The testicular response to hCG for progesterone and testosterone release was decreased in BPA-treated rats. Administration of BPA did not change the pituitary response to luteinizing hormone-releasing hormone (LH-RH) in castrated male rats treated with testosterone. Male sexual behavior also was not changed as a result of BPA treatment. These results suggest that BPA directly inhibits testicular functions and the increased level of plasma LH is probably due to a reduction in the negative feedback regulation by testosterone. The testis is probably a more sensitive site for BPA action than the hypothalamus-pituitary axis.

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It is becoming evident that many environmental chemicals bind to estrogen receptors, mimic or inhibit estrogenic actions, and may disturb endocrine systems in wildlife and humans (1–6). One such compound is bisphenol A (BPA), a monomer of polycarbonate plastics. BPA-based epoxy resins are widely used in consumer products, including composite dental sealants and the inner coating of food cans. BPA can be liberated from incompletely polymerized epoxy resins, and actually there have been several reports showing the presence of significant amounts of BPA in the liquid from canned vegetables (7) and in human saliva from patients treated with composite dental sealants (8). The estrogenic activities of BPA, which include binding to estrogen receptors, induction of progesterone receptors and promoting cell proliferation in estrogen-responsive MCF-7 breast cancer cells, have been demonstrated in *in vitro* systems. However, the potency of BPA was 3–4 orders of magnitude lower than that of estradiol (9). In addition to its estrogenic activities in MCF-7 cells, BPA stimulates prolactin (PRL) release in Fischer 344 rats (10), decreases plasma concentrations of testosterone in pubertal mice (11) and inhibits human chorionic gonadotropin (hCG)-stimulated steroidogenesis in mouse Leydig tumor cells (12). Other environmental xenobiotic estrogens, namely, alkylphenols, have also been reported to disrupt reproductive functions in rats (13,14).

It is clear that environmental xenobiotic estrogens such as bisphenol A and alkylphenols affect reproductive functions in experimental animals, and it has recently been hypothesized that exposure to estrogenic substances might account for the increasing frequency of infertility and the associated disorders of the male reproductive system in humans. However, there is little information on the effects of BPA on reproductive functions in adult males. In the present study, we determined the effects of BPA on adult male rat reproductive functions *in vivo* and investigated the sensitive site of BPA in the hypothalamus-pituitary-gonadal axis.

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Materials and Methods

Animals and Treatments. Adult male Wistar-Imamichi strain rats (300–350 g) were obtained from the Institute for Animal Reproduction (Ibaraki, Japan). Rats were housed under controlled temperature and lighting conditions (12 hr light:12 hr dark, light on at 7:00 AM) and supplied with food and water *ad libitum*. BPA (bisphenol A, Aldrich Chemical Co. Inc., Milwaukee, WI) was dissolved in 0.2 ml sesame oil and injected sc at 0800 h every day for 2 weeks. Five to six animals were used in each experiment.

Effects of BPA on Pituitary and Testicular Functions. To examine the effects of BPA on pituitary and testicular functions, five animals of each group were killed by decapitation at 0800 h 2 weeks after administration of BPA (1 mg/rat/day). The amount of BPA (1 mg) used in the present study was similar to the highest dose (8) of human exposure (931 μ g in saliva from patients treated with composite dental sealants) reported in some previous studies (7–9). This dose and the duration of BPA administration were determined by the results from preliminary experiment. We confirmed that daily administration of BPA (1 mg) for 1 week had no effects on pituitary and testicular functions. Blood was collected in heparinized tubes and centrifuged for the determination of plasma concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), PRL, testosterone, and inhibin. After decapitation, the pituitary and testes were removed and weighed. Pituitary glands and testes were homogenized in 0.05 M phosphate-buffered saline (PBS) at 4°C. The supernatant and plasma were collected and stored frozen at –20°C until assay for LH, FSH, PRL, testosterone, and inhibin.

Effects of BPA on the Testicular Response to hCG. To investigate the effects of BPA on testicular functions, hCG challenge was performed 2 weeks after administration of 100 μ g or 1 mg BPA. Twenty-four hours before the experiment, a cannula was inserted into the right atrium via the external jugular vein in each rat for drawing blood samples. Ten IU hCG or vehicle was injected through the atrial cannula, and blood samples were taken for plasma progesterone and testosterone before the injection and 30, 60, 90, 120, 150, and 180 min after administration of hCG. Blood was centrifuged, and the supernatant was stored frozen at –20°C until assay for progesterone and testosterone.

Effects of BPA on the Pituitary Response to LH-RH for LH Release. To examine the direct effects of BPA on pituitary function, especially LH secretion, LH–RH challenge was performed 2 weeks after administration of BPA. Adult male rats were castrated 5 days before the initiation of BPA treatment, and the castrated rats received sc administration of BPA (1 mg) and testosterone propionate (75 μ g) every day for 2 weeks. Twenty-four hours before the experiment, a cannula was implanted into the jugular vein in each rat for drawing blood samples. LH–RH (250 ng) dissolved in 0.2 ml of 50% polyvinylpyrrolidone or vehicle was injected subcutaneously. Blood samples for

plasma LH were taken from the cannula immediately before the injection and 0.25, 0.5, 1, 2, 3 and 4 hr after the injection. Blood was centrifuged, and the supernatant was stored frozen at –20°C until assay for LH.

Effects of BPA on Sexual Behavior. To examine the effects of BPA on the central nervous system, tests of copulatory behavior were conducted between 2000 and 2200 h under dim illumination from a 25-W red bulb, by direct observation of males paired with proestrous female rats in semicircular aquaria with sawdust bedding on the floor as described previously (15, 16). Two weeks after administration of BPA (1 mg), each male was allowed an adaptation period of 5 min prior to introduction of the stimulus female. Upon its introduction, each male was scored for the number of mounts, intromissions, and ejaculations. Subsequently, the following six parameters were calculated for 30 min of behavioral tests: (i) the mount latency, time from introduction of the female to the first mount; (ii) intromission latency, time from induction of the female to the first intromission; (iii) ejaculation latency, time from the first intromission of each ejaculatory series to ejaculation; (iv) mount frequency, the number of mounts per each ejaculatory series; (v) intromission frequency, the number of intromissions per each ejaculatory series; and (vi) ejaculation frequency, the number of ejaculations during the test period.

Radioimmunoassay (RIA). Concentrations of LH, FSH, and PRL were measured using National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) rat radioimmunoassay (RIA) kits for rat LH, FSH, and PRL. Hormones for iodination were rat LH-I-7, rat FSH-I-7, and rat PRL-I-5. The antisera used were anti-rat LH-S-10, anti-rat FSH-S-11, and anti-rat PRL-S-6. The results are expressed in terms of NIDDK rat LH-RP-2, FSH-RP-2, and PRL-RP-3. The intra- and inter-assay coefficients of variation were 5.5% and 8.9% for LH, 4.3% and 10.3% for FSH, and 5.8% and 7.6% for PRL, respectively.

Inhibin (17), progesterone, and testosterone (18) were measured by double-antibody RIAs using ¹²⁵I-labeled radioligands as described previously. Antiserum to progesterone (GDN 337) was kindly provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, CO), and antiserum to testosterone (FKA-102) was obtained from Cosmo Bio Co. (Tokyo, Japan). The intra- and inter-assay coefficients of variation were 3.7% and 6.4% for inhibin, 6.9% and 11.2% for progesterone, and 6.2% and 7.4% for testosterone, respectively.

Statistical Analyses. All results were expressed as the mean $\pm$ SEM. The data for the hormone levels in plasma, pituitary, and testes were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's protected least-significant difference (PLSD) test. Behavioral data were analyzed using Mann–Whitney *U*-tests. The effects of hCG challenges were analyzed using two-way ANOVA followed by Fisher's PLSD test; a value of *P* < 0.05 was considered significant.

Results

Effects of BPA on Pituitary and Testicular Functions. Plasma concentrations of LH and PRL were markedly increased by administration of BPA (1 mg) for 2 weeks (Fig. 1a,b). Pituitary contents of LH and PRL were also increased in BPA-treated rats compared to control rats, although the effects of BPA were not statistically significant (Fig. 1c,d). FSH levels in plasma and pituitary were not changed after administration of BPA (data not shown).

Plasma concentrations of testosterone and testicular contents of inhibin were significantly decreased (Fig. 2a,d), whereas testicular contents of testosterone and plasma concentrations of inhibin were not changed in BPA-treated rats compared to control rats (Fig. 2b,c).

Effects of BPA on Testicular Response to hCG.

In response to hCG (10 IU), plasma concentrations of progesterone and testosterone were markedly increased in the control group. Testicular responses to hCG for progesterone and testosterone release were significantly suppressed (Fig. 3b,d) as a result of treatment with high-dose BPA (1 mg), but administration of low-dose BPA (0.1 mg) did not affect either steroid (Fig. 3a,c).

Effects of BPA on the Pituitary Response to Luteinizing Hormone-Releasing Hormone (LH-RH) for LH Release. Administration of BPA (1 mg) did not change plasma concentrations of LH at 0 time in castrated rats treated with testosterone (Fig. 4), although plasma concentrations of LH were increased in testes-intact rats as a result of BPA administration (Fig. 1a). In castrated rats, pituitary LH response to LH-RH also was not affected after administration of BPA.

Effects of BPA on Sexual Behavior. Tests of copulatory behavior were conducted using adult male rats treated with BPA (1 mg) for 2 weeks. Six parameters including the mount latency (control, 13.6 ± 3.75 min; BPA, 13.0 ± 6.27 min), intromission latency (control, 21.0 ± 6.37 min; BPA, 23.4 ± 12.29 min), ejaculation latency (control, 129.6 ± 29.85 min; BPA, 160.0 ± 43.47 min), mount frequency (control, 11.9 ± 3.34 ; BPA, 11.6 ± 3.19), intromission frequency (control, 4.7 ± 0.75 ; BPA, 3.8 ± 0.99), and ejaculation frequency (control, 3.6 ± 0.24 ; BPA, 3.4 ± 0.24) were calculated for 30 min of behavioral tests. Administration of BPA did not affect any of the parameters in male sexual behavior.

Discussion

In the present study, administration of BPA significantly increased plasma concentrations of LH in adult male rats. Pituitary content of LH was also slightly increased in BPA-treated rats. In contrast, plasma concentrations of testosterone were decreased by BPA treatment. Testicular contents of inhibin were also significantly decreased in BPA-treated rats compared to control rats, although plasma concentrations of inhibin were not changed in adult male rats after administration of BPA. It has been well demonstrated that testosterone and inhibin are synthesized in Leydig and Sertoli cells, respectively, in adult male rats (19, 20). These findings suggest that BPA affects testicular functions in terms of Leydig and Sertoli cell functions and that the increased levels of plasma LH might be due to a reduction in the negative feedback regulation by testosterone. In contrast to LH levels, FSH levels in plasma and pituitary were not

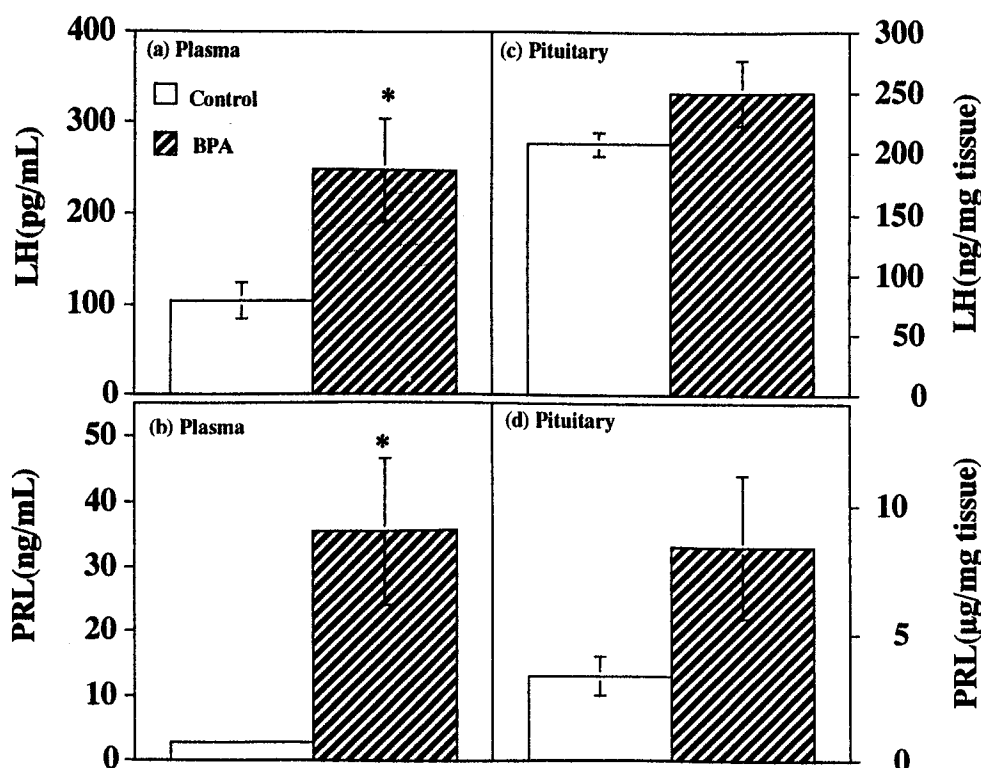


Figure 1. Plasma concentrations and pituitary contents of LH and PRL in adult male rats treated with BPA (1 mg) for 2 weeks. * $P < 0.05$ compared with control groups when analyzed using one-way ANOVA followed by Fisher's PLSD test.

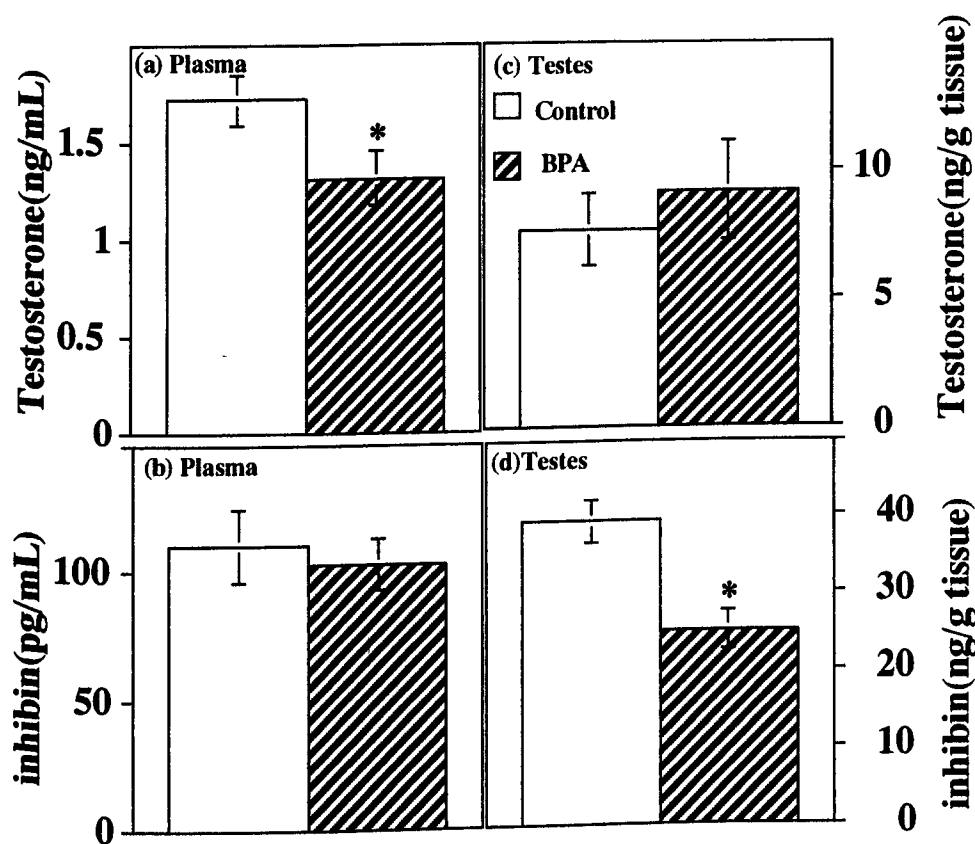


Figure 2. Plasma concentrations and testicular contents of testosterone and inhibin in adult male rats treated with BPA (1 mg) for 2 weeks. * $P < 0.05$ compared with control groups when analyzed using one-way ANOVA followed by Fisher's PLSD test.

changed by BPA administration. Since FSH secretion is mainly regulated by inhibin (21), the lack of any change in FSH may be due to the unchanged plasma concentrations of inhibin in BPA-treated rats in the present study. Administration of BPA increased PRL secretion in adult male rats. This effect of BPA is probably the direct action to anterior pituitary. Estrogen is well known to stimulate PRL secretion by acting at the pituitary level (22, 23). A previous report has shown that BPA increased *in vivo* and *in vitro* PRL release in estrogen-sensitive Fischer 344 rats but not in Sprague Dawley rats (10). These results support our findings in the present study, and the Wistar-Imamichi strain rats used in the present study might be sensitive to xenobiotic estrogen in terms of PRL secretion. Hyperprolactinemia has been shown to cause reproductive dysfunction (15, 24), but this dysfunction is not mediated *via* direct action on the testis. More likely it acts at the level of the hypothalamus-pituitary to inhibit LH-RH and LH secretion (24, 25). Inhibition of LH secretion has been reported during hyperprolactinemia (26, 27). In the present study, LH secretion was not decreased, but rather it was elevated in response to BPA, suggesting an action of BPA directly on the testis to inhibit testosterone secretion.

In the hCG challenge test to examine testicular (Leydig) functions in detail, administration of BPA significantly inhibited the testicular response to hCG for progesterone and testosterone release. It has been shown that BPA decreased hCG-stimulated cAMP and progesterone production in cultured mouse Leydig tumor cells by inhibiting LH

receptor-mediated signal transduction (12). This report indicated that the disturbance in signal transduction might be located between LH receptor and the adenylate cyclase, and that the effects of BPA were not related to estrogenic action as estradiol and diethylstilbestrol did not inhibit hCG-stimulated cAMP production (12). These results from the *in vitro* experiments agree with our *in vivo* results in the present study. The inhibition of hCG stimulated testosterone and progesterone release observed in BPA-treated rats may be due to the disturbance in the signal transduction between LH receptor and the adenylate cyclase. To examine the direct effects of BPA on LH secretion, LH-RH challenge was performed using castrated adult male rats treated with testosterone in the present study. Administration of BPA did not affect the plasma concentration of LH at time zero in castrated rats treated with testosterone, although plasma concentrations of LH were increased in testes-intact rats by BPA administration. In castrated rats, the pituitary response to LH-RH for LH release also was not affected after administration of BPA. These results suggest that BPA has little direct action on gonadotroph and the increased levels of plasma LH observed in BPA-treated intact rats are due to a reduction in the negative feedback regulation by testosterone.

In the present study, administration of BPA did not affect any of the parameters in male sexual behavior. The regulation of masculine sexual behavior is a complex process, and testosterone is one of most important factors for promoting it (28). The inhibition of testosterone secretion in

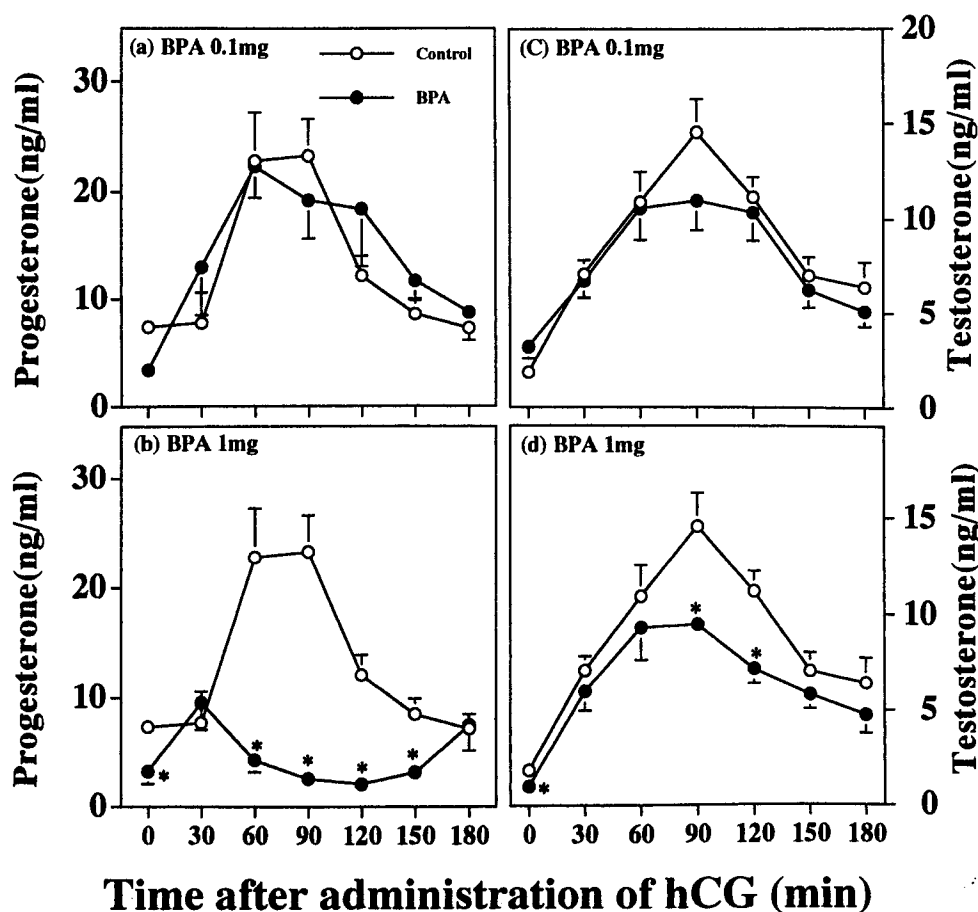


Figure 3. Effects of BPA on the testicular response to hCG for progesterone and testosterone release in adult male rats. Animals received sc daily administration of BPA (0.1 or 1 mg) for 2 weeks. Plasma concentrations of progesterone and testosterone were measured after a single iv injection of hCG (10 IU). * $P < 0.05$ compared with control when analyzed using two-way ANOVA followed by Fisher's PLSD test.

BPA-treated rats may have been not strong enough to interfere with male copulatory behavior in the present study and prolonged administration of BPA (1 mg) might be necessary to affect male sexual behavior.

The amount of BPA (1 mg) used in the present study was almost same as the highest dose (8) of human exposure (931 μg in saliva from patients treated with composite dental sealants) reported in some previous studies (7–9), but this amount was a high dose for adult male rats weighing 300–350 g. Animals received sc injection of BPA in the present study because 80% of the administered BPA was eliminated in fecal and 11% in urine within 3 days after its oral administration (29, 30). However, humans and animals are usually exposed to BPA orally. Experiments for the comparison with different routes of administration will be needed to fully ascertain the effects of BPA on male reproductive functions.

In conclusion, BPA directly inhibits testicular functions and increases plasma levels of LH. This increased level of LH is due to a reduction in the negative feedback regulation by testosterone, and the testis may be a more sensitive site for BPA than the hypothalamus–pituitary axis.

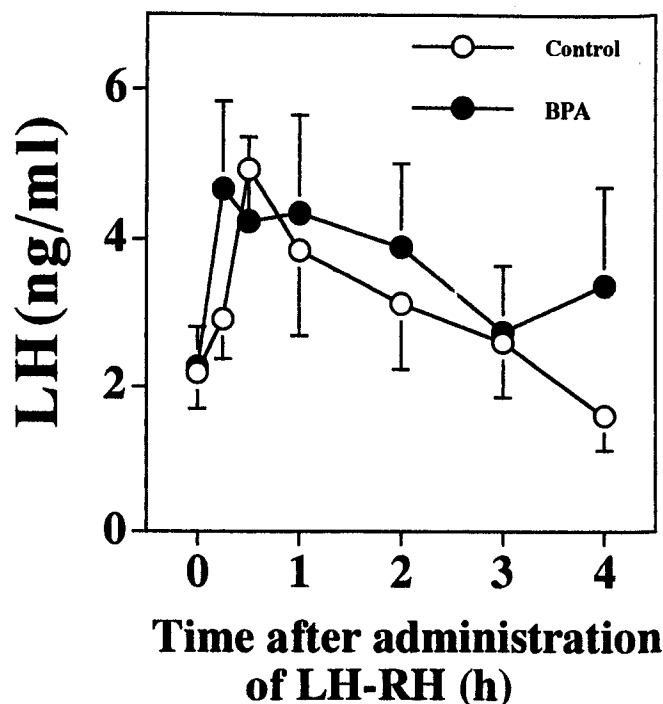


Figure 4. Effects of BPA on the pituitary response to LH-RH for LH release in castrated adult male rats treated with testosterone (75 μg). Castrated male rats received sc daily administration of BPA (1 mg) and testosterone (75 μg) for 2 weeks. Plasma concentrations of LH were measured after a single sc injection of LH-RH (250 ng) dissolved in 50% polyvinylpyrrolidone.

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