Endocrine, *vol. 25, no. 2, 163–172, November 2004* 0969–711X/04/25:163–172/$25.00

Adverse Effects of Environmental Toxicants, Octylphenol and Bisphenol A, on Male Reproductive Functions in Pubertal Rats

Chandana B. Herath,[1,†] Wanzhu Jin,[1,2] Gen Watanabe,[1,2]
Koji Arai,[3] Akira K. Suzuki,[4] and Kazuyoshi Taya[1,2]

[1]Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, Tokyo 183-8509; [2]United Graduate School of Veterinary Sciences, Gifu 501-1193; [3]Laboratory of Tissue Physiology, Tokyo University of Agriculture and Technology, Tokyo 305-8509; and [4]Air Pollutants Health Effects Research Team, National Institute of Environmental Studies, Ibaraki 305-0053, Japan. [†]Present address: Department of Medicine, University of Melbourne, Austin and Repatriation Medical Center, Heidelberg, Victoria 3084, Australia

It has been proposed that a global decline in sperm counts, semen quality, and several male reproductive disorders are associated with exposure to environmental chemicals. Thus, the present study examined the effects of two estrogenic chemicals, octylphenol (OP) and bisphenol A (BPA), on epididymal sperm counts and sperm motility, luteinizing hormone (LH)–releasing hormone (LHRH)–stimulated plasma LH and steroid hormones, insulin-like growth factor I (IGF-I), and accessory reproductive organs in pubertal male Wistar rats. Fifty-day-old rats in the OP group (n = 11) and BPA group (n = 11) received daily sc injections of the respective chemical at a dose of 3 mg/kg bw dissolved in 0.2 mL DMSO. Rats in the control group (DMSO group; n = 10) received 0.2 mL DMSO alone. After 2 wk of treatment, a jugular blood sample was taken, and, on the next day, a second blood sample was taken 1 h after an sc injection of LHRH (250 ng). After 5 wk of treatment, rats were deeply anesthetized and heart blood was collected. Epididymal sperm motility and sperm head counts were determined. LHRH increased plasma LH to higher levels in all groups, but the increases were significant ($p < 0.01$) in the BPA and OP groups. However, despite higher LH levels after LHRH injection, the incremental responses of testosterone and progesterone in the OP and BPA groups were small compared to those in the DMSO group, which showed a small LH response. After 5 wk of treatment, plasma testosterone levels were significantly ($p < 0.01$) reduced in the OP and BPA groups and this was accompanied by reduced ($p < 0.05$) epididymal sperm counts. However, the chemical-treated groups had high basal progesterone levels. No significant effects of chemicals on sperm motility parameters were noted. The chemical-induced increases ($p < 0.05$) of the weight of ventral prostate gland were coincided with elevated plasma IGF-I levels in the BPA ($p < 0.05$) and OP ($p < 0.01$) groups. The present results demonstrated that OP and BPA can reduce sperm counts resulting from lowered plasma testosterone in male rats just after puberty. The enlarged ventral prostate gland may possibly be associated with increased plasma IGF-I, raising the possibility of a link between these chemicals and prostate diseases because IGF-I has been implicated in the pathogenesis of human prostate cancers.

Key Words: Bisphenol A; octylphenol; sperm; testosterone; IGF-I; prostate.

Introduction

It has been proposed that a global decline in sperm counts and the semen quality is associated with exposure to environmental chemicals, in particular those with estrogenic activity *(1)* and reviewed by Toppari et al. *(2)*. The reports indicated that the reduction in mean sperm counts in some men has been estimated to be around 20–50% over the past 50 yr. Previous studies have confirmed that many of these man-made chemicals with estrogenic activity bind to estrogen receptors, mimic estrogenic action, thus leading to a disruption of vital endocrine system *(3–5)*. Two such chemicals that have been widely studied for their estrogenic potency both in vivo and in vitro are *p-tert*-octylphenol (OP) [*p*-(1,1,3,3-tetramethylbutyl)-phenol and Bisphenol A (BPA) [*p*, *p'*-isopropyridenebiphenol] *(5–8)*.

OP is an alkylphenolic compound derived as one of the major biodegradation products of non-ionic surfactants alkylphenol polyethoxylates (APEOs). These APEOs are released to the environment from the use of many chemicals such as detergents, paints, plastics, herbicides, and pesticides, and have become a major component in wastewater

Received September 14, 2004; Revised November 5, 2004; Accepted November 8, 2004.

Author to whom all correspondence and reprint requests should be addressed: Kazuyoshi Taya, D.V.M., Ph.D. Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan. E-mail: taya@cc.tuat.ac.jp

systems *(4,9)*. Bisphenol A (BPA), on the other hand, is a chemical primarily used as a monomer in the manufacture of numerous chemical products such as polycarbonate plastics and epoxy resins for use in the food packaging industry *(10)*. It has also been discovered that BPA is released in considerably larger quantities in the mouth in patients treated with plastic dental sealant *(11)*. In addition, concentrations of BPA in human maternal and fetal plasma and in the placenta at term were found to be comparable to a range that was shown to be toxic to reproductive organs of male and female animal offspring *(12)*. Because of large quantities of alkylphenols and BPA are manufactured every year to provide substrate for the production of APEOs, and polycarbonate plastics/epoxy resins, respectively, they continue to contaminate the environment along with their respective monomeric forms of the chemicals.

It has been shown that at higher doses OP can severely reduce the size and/or function of all of male gametogenic and accessory reproductive organs, alter the release of pituitary and gonadal hormones, and disrupt sperm production in adult male rats *(13)*. Male rat offspring born to the mothers exposed to a low dose of OP during gestation and lactation *(1)* or male mouse offspring born to the mothers exposed to either OP or BPA during gestation *(6)* had reduced testicular size and/or sperm production in adulthood. Similarly, either direct or indirect exposure of neonatal male rats to nonylphenol, another alkylphenolic compound with weak estrogenic activity, has been reported to adversely affect various reproductive parameters including a decrease in the size of the testes and male accessory organs that were associated with a reduced sperm count and sperm motility *(14,15)*.

Although the developmental abnormalities of male reproductive system have been linked mainly to *in utero* exposure of estrogen-mimicking chemicals, exposure to these chemicals during postnatal life may equally pose a threat to the health of human and animals. However, there has been no report to date comparing the adverse effects of both OP and BPA on luteinizing hormone–releasing hormone (LHRH)–stimulated steroid hormone profiles, and the effect on sperm motility parameters in male rats immediately after puberty, as this stage may be considered as one of highly sensitive periods to subtle effects of these chemicals during sexual maturation. In addition, no data available to examine a possible connection between such chemicals, the size of ventral prostate gland and plasma insulin-like growth factor-I (IGF-I) levels because IGF-I has been implicated in the pathogenesis of androgen-independent prostate cancer and hyperplasia, the prevalence rate of which is reported to be high in aged men *(16)*. Thus, there is a need for additional in vivo studies to investigate the possible connection between exposure to these chemicals and adverse effects on male reproductive health.

In the present study we examined the effects of a relatively low dose of OP and BPA on plasma concentrations of steroid hormones, immunoreactive inhibin (ir-inhibin), and IGF-I, basal and LHRH-stimulated luteinizing hormone (LH) and plasma steroids, and the effects on the weight of testes, accessory reproductive organs, sperm motility, and epididymal sperm counts.

Table 1
Mean Body Weights and the Weights of Testes and Accessory Reproductive Organs of Rats in Each Treatment Group after 5 wk of Treatment with Either DMSO or Chemicals[a]

	Mean weight (±SEM) (g)		
Description	DMSO	BPA	OP
Body weight	344 ± 6	348 ± 6	343 ± 5
Testes	1.35 ± 0.02	1.35 ± 0.02	1.34 ± 0.02
Seminal vesicles	1.6 ± 0.08	1.61 ± 0.08	1.52 ± 0.07
Right epididymis	0.48 ± 0.02	0.5 ± 0.01	0.45 ± 0.01†
Ventral prostate	0.42 ± 0.03	0.54 ± 0.03**	0.51 ± 0.03*

[a]DMSO: Dimethyl sulfoxide (control group; $n = 8$). BPA: Bisphenol A ($n = 10$), OP: Octylphenol ($n = 11$). Note that wet weight of seminal vesicles is given.

**$p < 0.01$, *$p < 0.05$, significantly different from corresponding DMSO group.

$^{\dagger}p < 0.05$, significantly different from corresponding BPA group.

Results

The rats in all treatment groups grew normally and there was no difference of the body weights between the groups (Table 1).

Effects of Exogenous LHRH on LH, Testosterone, and Progesterone

After 2 wk of chemical treatment, blood was collected before and after LHRH injection to investigate the effects of the chemicals on pituitary and gonadal responses (Fig. 1). Repeated measures analysis showed that LHRH-induced effect ($p < 0.001$) and the interaction between the chemicals and LHRH ($p < 0.01$) on LH response were highly significant. Before LHRH injection, a reduction in plasma LH concentrations was observed in the OP ($p < 0.05$) and BPA ($p < 0.06$) groups compared with that in the DMSO group. LHRH-stimulated rise of LH in the DMSO group was not statistically significant; however, the increases in both the BPA ($p < 0.01$) and OP ($p < 0.001$) groups were highly significant. The mean incremental response of LH following LHRH injection was higher in the OP group (Fig. 1A), but did not achieve statistically significant levels, apparently due to a higher biological variation.

Plasma testosterone concentrations were not different between the groups before LHRH injection. However, LHRH injection caused a significant elevation ($p < 0.001$) of plasma testosterone in the DMSO and BPA groups, but not in the OP group (Fig. 1B). Repeated measures analysis showed

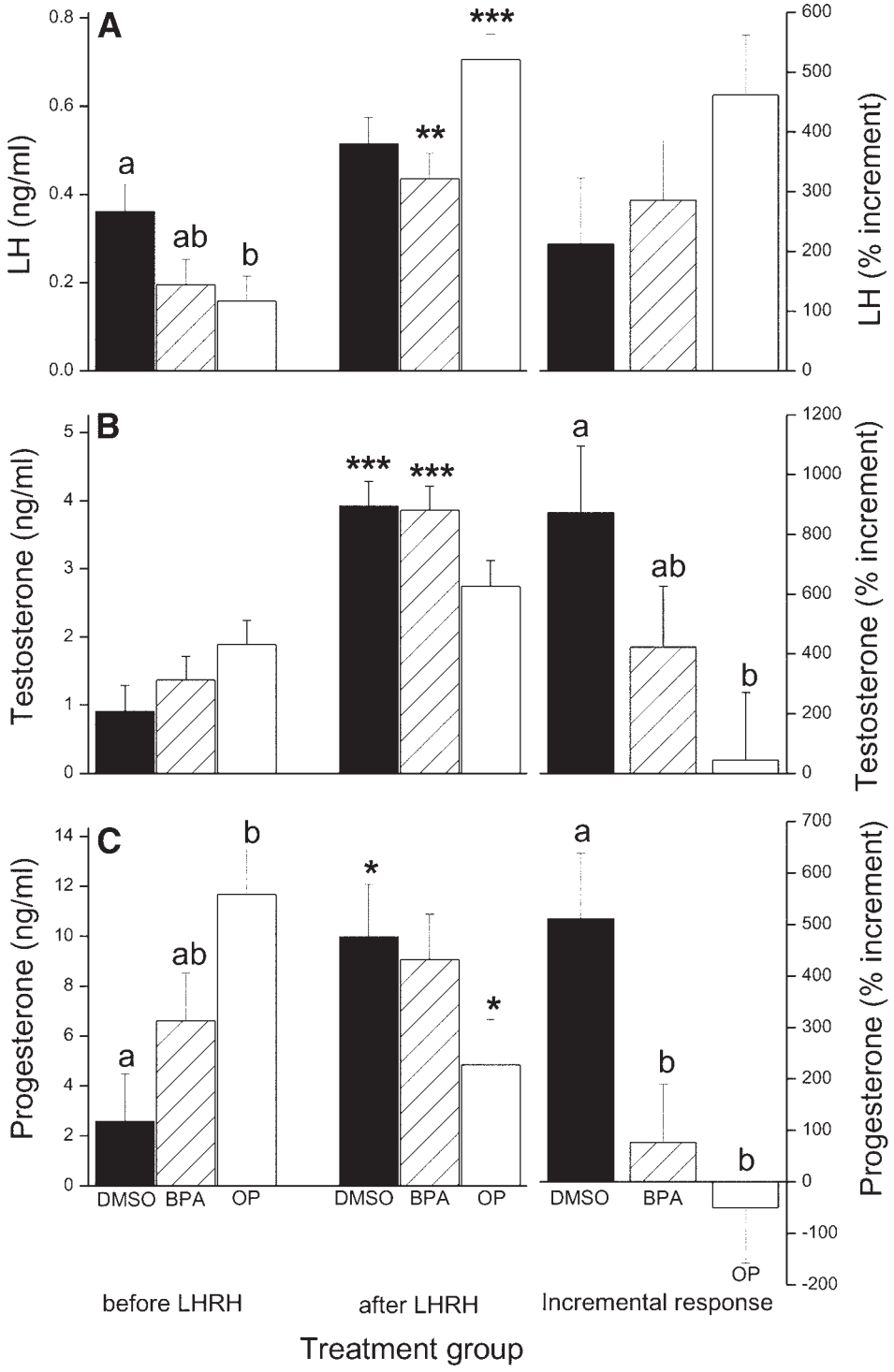

Fig. 1. Plasma concentrations of luteinizing hormone (LH) (**A**), testosterone (**B**), and progesterone (**C**) before and after luteinizing hormone–releasing hormone (LHRH) injection (250 ng, sc) in pubertal male rats treated with either dimethyl sulfoxide (DMSO, solid bars) as a control group or bisphenol A (BPA, hatched bars) or octylphenol (OP, open bars) administered as daily sc injections for 2 wk. Jugular blood was drawn at 15:00 h on the first day, and on the next day, LHRH was injected at 14:00 h and jugular blood was drawn at 15:00 h. LHRH-stimulated percentage increments of the respective hormonal response are shown at far right corner of each panel. The BPA and OP were administered at 3 mg/kg bw. Each *bar* represents the mean ± SEM concentration of each hormone or mean ± SEM incremental response from nine to eleven rats per treatment group. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ when compared to pre-LHRH injection levels within the same group. Means with different superscript letters (i.e., a, b) are significantly ($p < 0.05$) different when compared among different treatment groups before LHRH injection, and also within the incremental responses.

highly significant effects of LHRH ($p < 0.001$) and the interaction ($p < 0.01$) between LHRH and the chemicals on plasma testosterone concentrations. The mean incremental responses of plasma testosterone were lower in both BPA and OP groups compared with that in the DMSO group. However, only OP group showed a statistically significant ($p < 0.05$) reduction in testosterone incremental response (Fig. 1B).

Plasma progesterone concentrations before LHRH injection were higher in the BPA and OP groups compared to those in the DMSO group; however, the difference between the concentrations of OP and DMSO groups was significant ($p < 0.001$) (Fig. 1C). In a marked contrast to plasma testosterone responses, LHRH increased plasma progesterone concentrations to significantly ($p < 0.05$) higher levels only in the DMSO group but not in the BPA and OP groups. In fact the concentrations in the OP group fell significantly ($p < 0.05$) following LHRH injection. Repeated measures analysis showed significant effects of the chemicals ($p < 0.05$), and the interaction ($p < 0.01$) between the chemicals and LHRH on plasma progesterone concentrations. The mean incremental responses of plasma progesterone in all groups showed a similar pattern to that of plasma testosterone with lower responses in both BPA ($p < 0.05$) and OP groups ($p < 0.01$) compared with that in the DMSO group (Fig. 1C).

Effects on Testes, Accessory Reproductive Organs and Plasma Hormones

Body weight and the weights of testes and accessory reproductive organs are shown in Table 1. Weights of testes and seminal vesicle (wet weight) were not affected by the chemical treatment. However, OP caused a significant ($p < 0.05$) reduction in right epididymal weight compared to that in the BPA group. In marked contrast, interestingly, both BPA ($p < 0.01$) and OP ($p < 0.05$) treatments caused a significant increase in the size of ventral prostate gland.

After 5 wk of the chemical treatment, plasma concentrations of LH were not different between the groups (Fig. 2A). However, the treatment effect was highly significant ($p < 0.01$) on testosterone and thus plasma testosterone concentrations were significantly ($p < 0.01$) reduced in both the BPA and OP groups compared to the levels in the DMSO group (Fig. 2B). Plasma progesterone, on the other hand, was elevated and that the levels in the OP group were significantly ($p < 0.05$) higher than those in the DMSO group (Fig. 2C). Similarly, plasma IGF-I also elevated significantly in the BPA ($p < 0.05$) and OP ($p < 0.01$) groups compared to that in the DMSO group (Fig. 2D). Plasma ir-inhibin levels were not different between groups (data not shown).

Effects on Epididymal Sperm Count and Sperm Motility Parameters

After 5 wk of the treatment, the chemicals BPA and OP significantly ($p < 0.05$) reduced epididymal sperm head counts in chemical-treated groups in comparison with that in the rats treated with DMSO (Fig. 3). Data from computer-assisted sperm motility analysis are shown in Fig. 4. The effect of the chemicals on different sperm motility parameters as shown in Fig. 4 was not significant. However, there was a slight trend toward an increased number of slow-nonlinear sperms (Fig. 4A) and an increased number of slow-per-rapid motile sperms (Fig. 4B) in both BPA- and OP-treated rats when compared with those in the DMSO-treated group. In contrast, there was a slight reduction in the number of rapid-linear sperms in both BPA- and OP-treated rats when compared with those in the DMSO-treated rats (Fig. 4C). No trends of percentage motile sperm, curvilinear velocity, and straightline velocity were observed and therefore are not shown. Although a slight reduction in linearity index was noted in rats in both BPA and OP groups, it was not considered as a possible effect as other sperm velocity parameters were not changed.

Discussion

The present study demonstrates and confirms the previously published data that the environmental toxicants BPA and OP are capable of disrupting vital endocrine systems resulting in the reduction of sperm production in rats. There may be some adverse effects on sperm motility, although the differences observed in the present study were not statistically significant between the groups. It is also interesting to note that opposite to the depressive effects normally seen as a result of administration of environmental chemicals, BPA and OP at a dose of approx 9–15 ppm resulted in increased size of ventral prostate gland.

A small but significant reduction in basal LH was observed in rats treated with BPA and OP after 2 wk of treatment, although this effect had disappeared after 5 wk of chemical treatment. The effect observed after 2 wk of treatment in this study is opposite to the effect observed on LH in adult matured male rats given BPA for 2 wk *(17)*, possibly due to the difference in developmental stage of rats between the two studies. However, a previous study reported that BPA and OP reduced the amount of follicle-stimulating hormone (FSH) beta subunit in the pituitaries of the chemical-treated immature male rats *(18)*. In the present study, it is not known whether the two chemicals had a similar effect on LH synthesis. The LH lowering effect may be acute because a previous study did not find any difference in basal LH in mice treated orally with BPA for 4 or 8 wk *(19)*. Similar to plasma LH levels after 5 wk of treatment, we did not observe a difference in plasma inhibin after the same period of chemical treatment, and thus the effect on inhibin may be similar to that observed for plasma LH with no observable effect after 5 wk. In the present study, LHRH administration caused significant increases of plasma LH in rats in the BPA and OP groups, although an incremental response appeared prominent only in the OP group. The

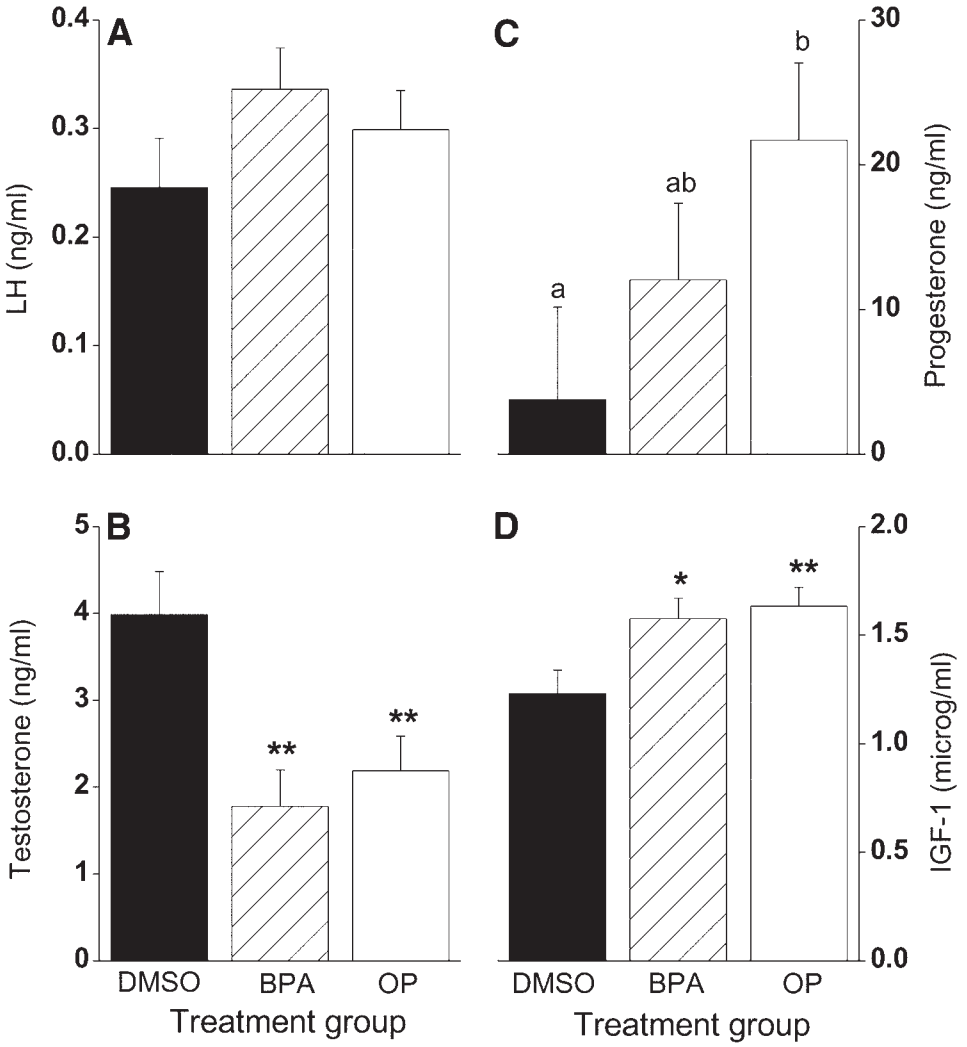

Fig. 2. Plasma concentrations of luteinizing hormone (LH) (**A**), testosterone (**B**), progesterone (**C**), and insulin-like growth factor I (IGF-I) (**D**) in pubertal male rats treated with either dimethyl sulfoxide (DMSO, solid bars) as a control group or bisphenol A (BPA, hatched bars) or octylphenol (OP, open bars) administered as daily sc injections for 5 wk. The BPA and OP were administered at 3 mg/kg bw. Each *bar* represents the mean ± SEM concentration of each hormone from 7 to 11 rats per treatment group. $^{**}p < 0.01$, $^{*}p < 0.05$ when compared to DMSO group. Means with different superscript letters (i.e., a, b) are significantly ($p < 0.05$) different when compared among different treatment groups.

mechanism by which the chemicals have affected LHRH-induced LH is not known. One possibility is that OP- and BPA-induced reduction of inhibin levels after 2 wk of chemical treatment *(17,18)* favored the increased secretion of LHRH-stimulated LH because inhibin has been shown to suppress LHRH-stimulated LH release *(20–22)* and upregulation of LHRH receptor by LHRH itself *(23)* in vitro. Age of our rats at the time of LHRH experiment was nearly same as the age of rats used by Kotsuji et al. *(21)* for obtaining pituitary cells. In addition, we have previously shown that plasma inhibin has a strong negative association with plasma LH in male rats *(24)* and plasma inhibin has a suppressive effect on LH secretion in the cyclic female rat *(25)*.

It is well known that pituitary LH is the major stimulus for testicular production of steroid hormones *(26,27)*. In the present study, higher plasma LH levels sustained by exogenous LHRH, increased plasma testosterone levels in all groups except the OP group in which the increase was not significant. These findings suggest that BPA and OP did not shut down steroidogenesis. However, in spite of a small incremental LH response following LHRH administration, plasma testosterone levels were highest in the DMSO group. Conversely, plasma testosterone increments in the BPA and OP groups following LHRH treatment were found to be lower than that in the DMSO group despite relatively higher incremental LH responses in the two chemical-treated groups. This suggests that though not completely affected, the chemicals BPA and OP had adversely affected steroidogenesis, possibly through their effects at different steps of steroidogenic pathway. It has been demonstrated that

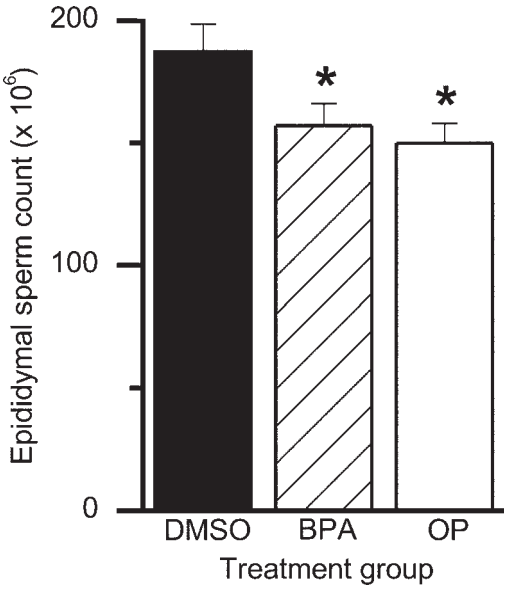

Fig. 3. Epididymal sperm head counts in pubertal male rats treated with either dimethyl sulfoxide (DMSO, solid bars) as a control group or bisphenol A (BPA, hatched bars) or octylphenol (OP, open bars). The BPA and OP were administered at 3 mg/kg bw as daily sc injections and after 5 wk, the rats were deeply anesthetized and right epididymis removed, weighed, and homogenized in 25 mL 0.85% NaCl (w/v) containing 0.05% Triton X-100 to measure sperm head counts by a hemocytometer. Each *bar* represents the mean ± SEM sperm head count of each group from 5 to 10 rats per treatment group. *$p < 0.05$ when compared to DMSO group.

environmental estrogenic chemicals such as OP can decrease human chorionic gonadotropin (hCG)-stimulated testosterone production by interfering at several steps of the steroidogenic pathway and raised the possibility that this effect of OP is apparently mediated through some other pathways other than classic estrogen receptor alpha and beta pathways *(28–31)*.

Progesterone, on the other hand, showed differential response as a result of elevated LH levels following LHRH administration. Basal progesterone levels were elevated in both BPA and OP groups. This agrees with previous reports that demonstrated a relatively low dose of BPA *(30,32)* and OP *(33)* stimulated basal progesterone production by rat and mouse Leydig cells and rat granulosa cells, respectively. However, in response to LHRH-stimulated high LH, plasma progesterone levels were increased only in the DMSO group but not in the BPA group. In sharp contrast, despite its highest LH response, plasma progesterone levels in the OP group fell by more than 50% of its basal levels. It has been shown that hCG-stimulated progesterone production was inhibited in cultured mouse or rat Leydig cells that had been preincubated with either BPA or OP *(29,31)*. The differential responses observed between the two chemical groups on plasma testosterone and progesterone may probably be due to their different estrogenic potencies with OP being

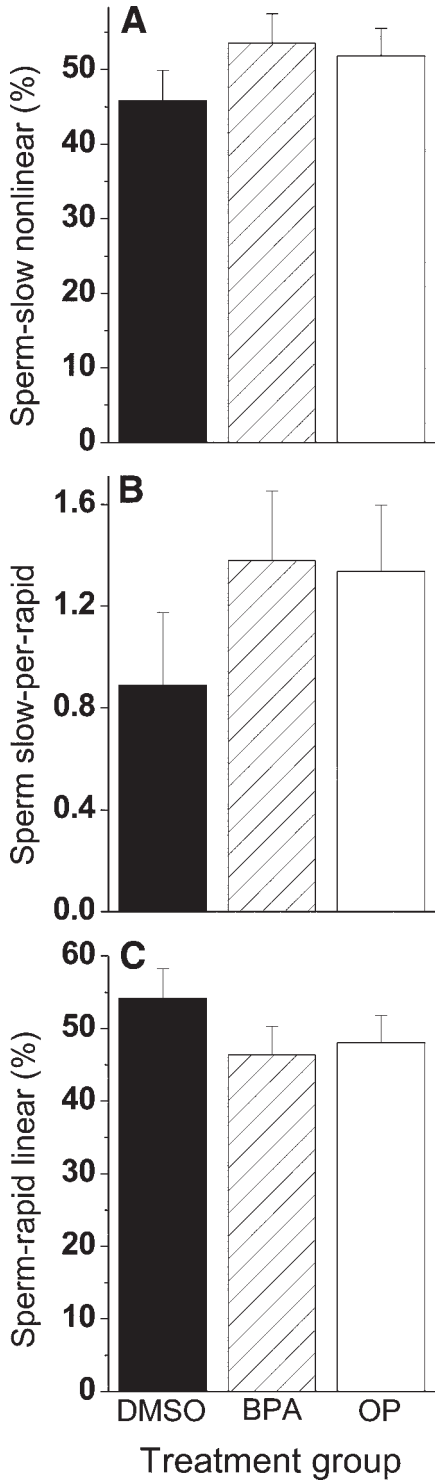

Fig. 4. Epididymal sperm motility parameters in pubertal male rats treated with either dimethyl sulfoxide (DMSO, solid bars) as a control group or bisphenol A (BPA, hatched bars) or octylphenol (OP, open bars). The BPA and OP were administered at 3 mg/kg bw as daily sc injections and after 5 wk, the rats were deeply anesthetized and the sperm motility parameters were measured with C. IMAGING computer-assisted sperm motility analysis system using sperms collected from left cauda epididymis. The parameters shown are slow-nonlinear (**A**), number of sperms showing slow motility per rapid-motile sperm (**B**), and rapid linear (**C**). Each *bar* represents the mean ± SEM from 9 to 11 rats per treatment group.

comparatively more potent than BPA *(4)* or, alternatively, it may be due to a difference in non-estrogen receptor mediated pathways *(28–30)*.

Both BPA and OP caused a significant reduction in sperm numbers in the epididymis and thus, agree with a number of previous reports that demonstrated the adverse effects of low doses of these chemicals on sperm production *(6, 34)*. The reduction in sperm numbers in both BPA and OP groups was accompanied by more than 50% reduction in their plasma testosterone levels, suggesting that disruption of basal testosterone production led to a reduction in the sperm numbers. A previous report demonstrated that a dose of OP slightly higher than that used in the present study progressively decreased hCG-stimulated testosterone production to about 40–80% below control levels in cultured testicular cells *(28,29)*. The reduction in testosterone production in rats treated with BPA and OP is possibly due to their interference in the steroidogenic pathway as shown by several previous reports *(18,28–31)*. In addition to the effect via lowered testosterone, a direct toxic effect on spermatogenic and Sertoli cells cannot be ruled out as a previous report indicated that low doses of OP were toxic on the viability of these cells collected from rats and cultured in vitro *(35)*. Similarly, peripubertal mice treated orally with BPA had direct toxic effects with lesions found in the seminiferous tubules *(19)*. A comparison between testosterone levels after 2 wk and 5 wk of chemical treatment reveals that basal hormone levels were not changed in both the BPA and OP groups. This indicates that rats in the DMSO group increased testosterone biosynthesis as they matured, but, on the contrary, this did not happen in the rats treated with BPA and OP resulting in the disruption of sperm production. On the other hand, both chemicals were stimulatory on plasma progesterone levels, and it was apparent that after 5 wk of treatment the levels had rose approximately as twice as those found after 2 wk, probably indicating the persistent effect of the chemicals on steroid biosynthesis whereas there was no any drastic change in its level in the DMSO group.

There has been no report to date that demonstrated the effects of both BPA and OP on sperm motility in pubertal rats. Thus the present study investigated different sperm motility parameters and found that at 9–15 ppm range there were no significant effects of the chemicals on the parameters analyzed. It should be noted, however, that there were some trends on some of the motility parameters in a manner that is similar in both the BPA and OP groups. For example, the percentage of rapid motile sperms showed a downward trend in comparison with that in the DMSO group. On the other hand, the percentage of slow-nonlinear sperms and the ratio of rapid-to-slow sperms showed an upward trend in comparison with those in the DMSO group. In support of these findings, however, Chitra et al. *(36)* have reported BPA-induced reduction in sperm motility. Thus, further studies are warranted to confirm the adverse effects of BPA and OP on various sperm motility parameters.

The chemicals did not have any effect on normal body growth. However, the present doses of both BPA and OP affected the weight of accessory reproductive organs. On the epididymis, OP was inhibitory and suppressed the size of the gland. On the ventral prostate, on the other hand, both chemicals were stimulatory. The suppressive effect of OP on epididymis has previously been reported *(13)*. Higher doses of BPA are reported to suppress the prostate gland *(37)*. It has been shown previously that pregnant mice fed with low doses of BPA gave male offspring with enlarged prostate gland *(38,39)*. Thus, natural estrogen such as estradiol, artificial estrogen such as diethylstilbestrol (DES), and chemicals with estrogenic activity at higher doses inhibit the size of ventral prostate gland while at lower doses they increase the size of ventral prostate gland *(37–40)*. In the present study, it is not known whether the effect on the prostate is due to a direct action of the chemicals. It has been demonstrated that DES, a potent synthetic estrogen, and BPA stimulated the growth of 17-d-old fetal prostate in culture *(39,41)*, and that the effect was evident in the presence or absence of testosterone in the culture medium *(39)*. Moreover, although BPA and OP were without effect on the prostate in immature rats treated with the chemicals during first five days after birth *(42)*, male rats exposed to BPA on postnatal d 22–32 showed enlarged prostate when measured at 4 mo of age *(43)*, and an enlarged prostate has been observed in mature rats exposed to BPA *(36)*, reflecting that the sensitivity of prostate to such chemicals is high during fetal life but appears to change with effects depending on age after birth. On the other hand, in the present study, both chemicals increased plasma IGF-I concentrations. Source of this IGF-I is not known; however, estrogen has been shown to increase plasma IGF-I levels in rats *(44)*, wethers *(45)*, and humans *(46)*, and that the liver appears to be the major site of estrogen-induced IGF-I secretion in wethers *(45)*. Although the mechanism by which BPA and OP altered plasma IGF-I levels is not clear, the stimulatory effect on the somatotropic axis may occur through estrogen receptor pathways *(47,48)*. Thus, it is possible that, in the present study, liver is a potential site that contributed to the elevated plasma IGF-I levels in response to BPA and OP treatments. The chemical-induced ventral prostate enlargement may be due either to a direct action via estrogen receptor mediated pathway *(38)* or an indirect action via elevated plasma IGF-I. BPA has been shown to activate IGF-I gene expression and signaling pathway leading to epithelial cell proliferation in the mouse uterus *(49)*. A strong positive association has been observed between circulating IGF-I levels and prostate cancer risk in men aged 40 yr and above *(50)*. IGF-I has been implicated in the pathogenesis of androgen-independent prostate cancer and that inhibition of growth hormone–releasing hormone (GHRH) secretion by GHRH antagonists in mice implanted with PC-3 prostate tumors caused a decrease of plasma IGF-I, thus leading to a significant reduction of tumor mass *(16)*. However, a

role for prolactin, the secretion of which is shown to be induced by both BPA and OP, in the pathogenesis of prostate enlargement cannot be ruled out *(42,43)*. On the other hand, it has been reported that concentrations of an OP-related alkylphenolic compound 2,4-di-*tert*-pentylphenol were in the 10^{-3} *M* range in fat tissue in carp in the Detroit River's Trenton Channel *(51)*. This level is higher than that reported for other derivatives of alkylphenol polyethoxylates in drinking water, sewage sludge, and river water sediment *(9,52)* and well above the levels of the chemicals used in the present study, indicating that these alkylphenolic compounds have the ability to bioaccumulate in larger amounts in tissues. Moreover, concentrations of BPA in human maternal and fetal plasma and in the placenta at term were found to be comparable to a range that was shown to be toxic to reproductive organs of male and female animal offspring *(12)*. In light of above findings a possible link between environmental estrogenic chemicals such as BPA and OP and high prevalence rate of prostate-associated diseases in men can not be ruled out.

In conclusion, both chemicals differentially affect basal as well as LHRH-induced steroid production, and based on the present and previously reported data, the effects of BPA and OP on steroidogenesis appear to be varied depending upon the developmental stage. The chemicals at 9–15 ppm amounts can increase the size of ventral prostate gland and that this effect may be either direct or indirect via elevation of plasma IGF-I levels. The two chemicals are equally potent in exerting adverse effects, suggesting that they are capable of posing a threat to reproductive health. The difference between the two chemicals in exerting some of the effects could be due either to the differences in their estrogenic potencies or, alternatively, to a difference in non-estrogen receptor mediated pathways. It should be noted that there is a possibility that the absolute values for plasma hormones may have been confounded by the effect of ether-stress; however, the differences of hormone levels between the treatment groups may not be expected to suffer due to a presumably equal effect of ether on all groups. The present results also demonstrated that environmental chemicals such as BPA and OP can adversely affect sperm production in rats just after puberty. Further studies are warranted to confirm the possible effect of these chemicals on sperm motility parameters.

Materials and Methods

Animals

Forty two-day-old male Wistar–Imamichi rats were purchased from Imamichi Institute for Animal Reproduction, Ibaraki, Japan. They were housed in metal cages and maintained in a room with controlled illumination (14 h light : 10 h darkness and lights on at 05:00 h) and temperature (22–24°C), with free access to commercial pellets (Nosan Corporation, Yokohama, Japan) and given tap water *ad libitum*. The commercial pellets contain soy as one ingredient. The humidity of animal room varied from 55% to 75%. They were acclimatized during the next 8 d before the commencement of the experiments. All procedures were carried out in accordance with the guidelines established by the Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, for use of laboratory animals.

Chemicals

The OP (min 95% pure), BPA (min 95% pure), and dimethyl sulfoxide (DMSO) were purchased from Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Known amounts of OP and BPA were dissolved in DMSO, and these stock vials were then sealed and kept at room temperature during the experiments. Protective clothing such as gloves and apron was used during handling of these chemicals.

Experimental Protocol

When rats were 50 d old the experiments were begun. The dosage of OP and BPA used for injections in the present experiments was 3 mg/kg body weight (bw). Although the present dosage of the chemicals is relatively small compared with that used in typical toxicological studies, 3 mg/kg bw can be categorized as a pharmacological dose. Mean body weight of rats (n = 32) was 204 ± 6 (±SD) g at the commencement of the experiments. Ten to eleven rats were randomly assigned per treatment group. The rats in each treatment group received daily sc injections of either 0.2 mL DMSO alone (DMSO group = control group) or 0.2 mL DMSO containing appropriate amount of OP (OP group) or BPA (BPA group). Starting from day 1 of the experiment all the rats were weighed every 5 d during the injection period and the amount of OP and BPA to be injected into each rat was calculated accordingly. Based on weights, the amount of OP or BPA injected into each rat represented approx 9–15 ppm during the 5 wk of the experimental period.

Effects of Exogenous LHRH on LH, Testosterone, and Progesterone

To investigate the pituitary and gonadal hormonal response to LHRH, after 2 wk of chemical treatment, rats were bled by jugular venipuncture at 15:00 h under light ether anesthesia. We have consistently used ether as an anesthetic agent for laboratory animals and to measure plasma hormones in the past *(24)*. On the next day at 14:00 h, the rats were given an LHRH (250 ng, sc) injection dissolved in 0.2 mL of 50% polyvinylpyrrolidinone (50% PVP) and blood was drawn at 15:00 h. It was previously shown that a single injection of LHRH (250 ng, sc) dissolved in 0.2 mL of 50% PVP caused a clear time course increase of plasma LH with a maximum level reaching at around 1 h postinjection *(17,53)*. Moreover the pituitary responsiveness to LHRH for LH release was maintained in ether-stressed rats *(53)*. Therefore, 1 h after an sc injection of LHRH dissolved in 0.2 mL of 50% PVP was considered adequate to evalu-

ate the capacity of pituitary for LH release. The blood samples were used for determination of LH, testosterone, and progesterone.

Effects on Epididymal Sperm Count and Sperm Motility Parameters

After 5 wk of chemical treatment, each rat was kept for an extended period of time in a chamber containing ether vapor to deliver deep anesthesia. Deeply anesthetized rats were bled to death by heart puncture. Right epididymis was removed and homogenized (TOMY, SEIKO Co. LTD, Tokyo, Japan) in 25 mL 0.85% NaCl (w/v) containing 0.05% Triton X-100 to measure sperm head counts by a hemocytometer. The sperm motility parameters were measured with C. IMAGING computer-assisted sperm motility analysis system. Sperms from left cauda epididymis were incubated for 3 min at 37°C in 0.01 *M* medium buffer, pH 7.2. The medium buffer was made up of 59.8 mg of HEPES (Dojindo, Japan), 982 mg medium 199 (Biocell, CA, USA), 500 mg of bovine serum albumin (Wako, Japan), and 220 mg of $NaHCO_3$ (Wako) dissolved in 100 mL deionized water. After sperms were incubated in medium buffer at 37°C, an aliquot of the solution was then diluted 10- to 20-fold and 10 µL was placed onto the Micro Cell-HAC chamber, which has a depth of 50 µm (Conception Technologies, San Diego, CA, USA). Analyses of motility characteristics were performed on at least 200 cells for each sample. Sperm motility, as viewed on an Olympus microscope (4×, pseudo-dark filed optics) with a stage warmer (37°C) (MP10DM, Kitazato, Japan), was used by C. IMAGING system. The C. IMAGING system settings were as follows: frames analyzed = 15; framing rate = 30; maximum velocity = 1200 µm/s; threshold velocity = 45 µm/s; pixel scale 3.26 mm/pixel; maximum average number of cells / field = 30; cell size range = 350–1600 pixel. The following characteristics were analyzed: the percentages of motile, rapid linear and slow nonlinear spermatozoa, curvilinear velocity (the total distance traveled divided by the total time the cell was tracked), straightline velocity (straightline distance), linearity index (ratio of the straightline distance to the actual tracked distance), and a ratio of slow-per-rapid sperm.

Effects on Accessory Reproductive Organs and Plasma Hormones

Blood collected by heart puncture was used for determination of LH, progesterone, testosterone, ir-inhibin, and IGF-I. Weights of testes, seminal vesicles (wet weight), right epididymis and ventral prostate gland were recorded.

Blood Processing

All blood samples were collected into plastic tubes containing heparin (15 IU/mL blood) as anticoagulant to prevent clotting. The samples were stored in ice and centrifuged at 3300*g* for 15 min at 4°C immediately after completing the experiment. The resulting plasma was decanted and stored at –20°C, until assayed for LH, progesterone, testosterone, ir-inhibin and IGF-I concentrations.

Radioimmunoassays

Plasma concentrations of LH were measured using NIDDK radioimmunoassay (RIA) kit (Baltimore, MD, USA) for rat LH. The antiserum used was anti-rat LH (S-10)**.** Intra- and interassay coefficients of variation were 5.4% and 6.9%.

Plasma concentrations of progesterone and testosterone were determined by double-antibody RIAs using ^{125}I-labeled radioligands, as described previously *(54)*. Dr. G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO) kindly supplied antisera against progesterone (GDN#377) and testosterone (GDN#250). Intra- and interassay coefficients of variation were 3.5% and 13.4% for progesterone, and 6.8% and 17.1% for testosterone, respectively.

Plasma concentrations of IGF-I were determined after extraction using acid-ethanol-cryo precipitation method, as described previously *(55)*, with some modifications to RIA procedure. Standards were prepared from hIGF-1 (Lot # 126R01A C247, PEPROTECH EC LTD). The antiserum used was NIDDK polyclonal antiserum made against hIGF-1 (ID# AFP4892898). All samples were analyzed in a single assay and the intraassay coefficient of variation was 6.3%.

Plasma concentrations of ir-inhibin were measured by a double-antibody RIA as described previously *(56)*. Purified bovine inhibin (32-kilodalton) was used as standard. The assay system does not distinguish dimeric inhibin from the α subunit monomer. All samples were analyzed in a single assay and the intra-assay coefficient of variation was 9.8%.

Statistics

Statistical significance between the means of treatment groups was determined using ANOVA and Duncan's Multiple Range test. Repeated measures analysis was carried out for comparisons of LH, testosterone and progesterone individually at three different time points. Data are presented as least square means ± standard errors (±SEM). A probability value less than 0.05 was considered significant. All statistical analyses were carried out using the computer software package "SAS" (SAS Statistics, Version 6.11, SAS Institute Inc., Cary, NC, USA).

Acknowledgments

We are grateful to the National Hormone and Pituitary Program, NIDDK, NIH, MD and Dr. A. F. Parlow for rat LH RIA kit, and to Dr. G. D. Niswender for the generous gifts of testosterone and progesterone antisera. This work was supported by a grant-in-aid from the Japan Society for the Promotion of Science, and a Grant-in-Aid for Scientific Research (The 21st Century Center of Excellence Program, E-1) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Sharpe, R. M., Fisher, J. S., Millar, M. M., Joblin, S., and Sumpter, J. P. (1995). *Environ. Health Perspect.* **103,** 1136–1143.
2. Toppari, J., Larsen, J. C., and Christiansen, P., et al. (1996). *Environ. Health Perspect.* **104(Suppl 4),** 741–803.
3. Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L., and Feldman, D. (1993). *Endocrinology* **132,** 2279–2286.
4. White, R., Joblin, S., Hoare, S. A., Sumpter, J. P., and Parker, M. G. (1994). *Endocrinology* **135,** 175–182.
5. Steinmetz, R., Brown, N. G., Allen, D. L., Bigsby, R. M., and Ben-Jonathan, N. (1997). *Endocrinology* **138,** 1780–1786.
6. vom Saal, F. S., Cooke, P. S., Buchanan, D. L., et al. (1998). *Toxicol. Ind. Health* **14,** 239–260.
7. Fisher, J. S., Turner, K. J., Brown, D., and Sharpe, R. M. (1999). *Environ. Health Perspect.* **107,** 397–405.
8. Herath, C. B., Watanabe, G., Katsuda, S., Yoshida, M., Suzuki, A. K., and Taya, K. (2001). *Biol. Reprod.* **64,** 1216–1224.
9. Giger, W., Brunner, P. H., and Schaffner, C. (1984). *Science* **225,** 623–625.
10. Ben-Jonathan, N. and Steinmetz, R. (1998). *Trends Endocrinol. Metabol.* **9,** 124–128.
11. Olea, N., Pulgar, R., Perez, P., et al. (1996). *Environ. Health Perspect.* **104,** 298–305.
12. Schonfelder, G., Wittfoht, W., Hopp, H., Talsness, C. E., Paul, M., and Chahoud, I. (2002). *Environ. Health Perspect.* **110,** A703–A707.
13. Boockfor, F. R. and Blake, C. A. (1997). *Biol. Reprod.* **57,** 267–277.
14. Lee, P. C. (1998). *Endocrine* **9,** 105–111.
15. Lee, P. C., Arndt, P., and Nickels, K. C. (1999). *Endocrine* **11,** 61–68.
16. Plonowski, A., Schally, A. V., Varga, J. L., et al. (2000). *Prostate* **44,** 172–180.
17. Tohei, A., Suda, S., Taya, K., Hashimoto, T., and Kogo, H. (2001). *Exp. Biol. Med.* **226,** 216–221.
18. Saunders, P. T., Majdic, G., Parte, P., et al. (1997). *Adv. Exp. Med. Biol.* **424,** 99–110.
19. Takao, T., Nanamiya, W., Nagano, I., Asaba, K., Kawabata, K., and Hashimoto, K. (1999). *Life Sci.* **65,** 2351–2357.
20. Fukuda, M., Miyamoto, K., Hasegawa, Y., Ibuki, Y., and Igarashi, M. (1987). *Mol. Cell. Endocrinol.* **51,** 41–50.
21. Kotsuji, F., Winters, S. J., Keeping, H. S., Attardi, B., Oshima, H., and Troen, P. (1988). *Endocrinology* **122,** 2796–2802.
22. Farnworth, P. G., Robertson, D. M., de Kretser, D. M., and Burger, H. G. (1988). *J. Endocrinol.* **119,** 233–241.
23. Wang, Q. F., Farnworth, P. G., Findlay, J. K., and Burger, H. G. (1989). *Endocrinology* **124,** 363–368.
24. Herath, C. B., Watanabe, G., Wanzhu, J., et al. (2001). *J. Androl.* **22,** 838–846.
25. Arai, K., Watanabe, G., Taya, K., and Sasamoto, S. (1996). *Biol. Reprod.* **55,** 127–133.
26. Ewing, L. L., Wing, T. Y., Cochran, R. C., Kromann, N., and Zirkin, B. R. (1983). *Endocrinology* **112,** 1763–1769.
27. Valladares, L. E., Ronco, A. M., and Pino, A. M. (1986). *J. Endocrinol.* **110,** 551–556.
28. Murono, E. P., Derk, R. C., and de Leon, J. H. (1999). *Reprod. Toxicol.* **13,** 451–462.
29. Murono, E. P., Derk, R. C., and de Leon, J. H. (2000). *Reprod. Toxicol.* **14,** 275–288.
30. Murono, E. P. and Derk, R. C. (2002). *J. Steroid. Biochem. Mol. Biol.* **81,** 181–189.
31. Nikula, H., Talonpoika, T., Kaleva, M., and Toppari, J. (1999). *Toxicol. Appl. Pharmacol.* **157,** 166–173.
32. Song, K. H., Lee, K., and Choi, H. S. (2002). *Endocrinology* **143,** 2208–2215.
33. Nejaty, H., Lacey, M., and Whitehead, S. A. (2001). *Exp. Biol. Med.* **226,** 570–576.
34. Al-Hiyasat, A. S., Darmani, H., and Elbetieha, A. M. (2002). *Eur. J. Oral Sci.* **110,** 163–167.
35. Raychoudhury, S. S., Blake, C. A., and Millette, C. F. (1999). *Toxicol. Appl. Pharmacol.* **157,** 192–202.
36. Chitra, K. C., Latchoumycandane, C., and Mathur, P. P. (2003). *Toxicology* **185,** 119–127.
37. Takahashi, O. and Oishi, S. (2003). *Food Chem. Toxicol.* **41,** 1035–1044.
38. Nagel, S. C., vom Saal, F. S., Thayer, K. A., Dhar, M. G., Boechler, M., and Welshons, W. V. (1997). *Environ. Health Perspect.* **105,** 70–76.
39. Gupta, C. (2000). *Proc. Soc. Exp. Biol. Med.* **224,** 61–68.
40. vom Saal, F. S., Timms, B. G., Montano, M. M., et al. (1997). *Proc. Natl. Acad. Sci. USA* **94,** 2056–2061.
41. Gupta, C. (2000). *Urol. Res.* **28,** 223–229.
42. Khurana, S., Ranmal, S., and Ben-Jonathan, N. (2000). *Endocrinology* **141,** 4512–4517.
43. Stoker, T. E., Robinette, C. L., Britt, B. H., Laws, S. C., and Cooper, R. L. (1999). *Biol. Reprod.* **61,** 1636–1643.
44. Sahlin, L., Norstedt, G., and Eriksson, H. (1994). *J. Steroid. Biochem. Mol. Biol.* **51,** 57–66.
45. Johnson, B. J., White, M. E., Hathaway, M. R., Christians, C. J., and Dayton, W. R. (1998). *J. Anim. Sci.* **76,** 491–497.
46. Campagnoli, C., Biglia, N., Cantamessa, C., Lesca, L., Lotano, M. R., and Sismondi, P. (1998). *Gynecol. Endocrinol.* **12,** 259–266.
47. Huynh, H. T., Tetenes, E., Wallace, L., and Pollak, M. (1993). *Cancer Res.* **53,** 1727–1730.
48. Metzger, D. L. and Kerrigan, J. R. (1994). *J. Clin. Endocrinol. Metab.* **79,** 513–518.
49. Klotz, D. M., Hewitt, S. C., Korach, K. S., and Diaugustine, R. P. (2000). *Endocrinology* **141,** 3430–3439.
50. Chan, J. M., Stampfer, M. J., Giovannucci, E., et al. (1998). *Science* **279,** 563–566.
51. Shiraishi, H., Carter, D. S., and Hites, R. A. (1989). *Biomed. Environ. Mass Spectrom.* **18,** 478–483.
52. Clark, L. B., Rosen R. T., Hartman, T. G., et al. (1992). *Int. J. Environ. Anal. Chem.* **47,** 167–180.
53. Tohei, A., Tomabechi, T., Mamada, M., Akai, M., Watanabe, G., and Taya, K. (1997). *J. Vet. Med. Sci.* **59,** 329–334.
54. Taya, K., Watanabe, G., and Sasamoto, S. (1985). *Jpn. J. Ani. Reprod.* **31,** 186–197.
55. Blum, W. F. and Breier, B. H. (1994). *Growth Regul.* **4(Suppl 1),** 11–19.
56. Hamada, T., Watanabe, G., Kokuho, T., et al. (1989). *J. Endocrinol.* **122,** 697–704.