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A TWO-GENERATION REPRODUCTIVE TOXICITY STUDY OF BUTYL BENZYL PHTHALATE IN RATS

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ABSTRACT — A two-generation reproductive toxicity study with extra parameters was performed for Butyl benzyl phthalate (BBP). The compound was administered orally by gavage with the doses of 0, 100, 200, or 400 mg/kg/day to groups of 24 Crj:CD®(SD)IGS rats of both sexes to confirm the utility of the protocol for identification of non-steroid chemicals with endocrine activity by ssessing effects on parental animals and offspring. Softening of the testes, diffuse atrophy of testicular seminiferous tubules, decreased spermatozoa and/or residual germ cells in the epididymal lumina were observed in the F1generation after doses more than 100 mg/kg, lowering of the F1 epididymal weights at doses more than 200 mg/kg, along with low F0 epididymal weights, Leydig cell hyperplasia, residual germ cells in the epidimymal lumina, and low seminal vesicle weights, small testes and epididymes, partial aplasia or aplasia of the epididymes, and Leydig cell hyperplasia in the F1 generation with 400 mg/kg. With regard to effects on the reproductive capacity, F1 parents at the dose of 400mg/kg showed a reduced fertility index and delayed preputial separation of the penis. In the offspring, lowered body weights in the F1 case, and change in anogenital distance in the F1 females and F2 males were observed at doses more than 100 mg/kg, with low splenic weights at 400 mg/kg in both generations. Thus, the utility of this protocol was confirmed. In the parental animals, the no observed effect level (NOEL) and the no observed adverse effect level (NOAEL) were less than 100 mg/kg/day, and no serious effects on the reproductive capacity were induced at doses less than 200 mg/kg/day. The NOEL and NOAEL for the growth and development of offspring were concluded to be less than 100 mg/kg/day.

KEY WORDS: Butyl benzyl phthalate, BBP, Two-generation reproductive toxicity study, Rats

INTRODUCTION

Butyl benzyl phthalate (BBP), used as a plastic plasticizer (for floors, walls, tiles; paste; artificial leather; and upholstery), is known to bind to estrogen receptors, although only very weakly (Jobling *et al.*, 1995; Hashimoto *et al.*, 2000; Ministry of Economy, Trade and Industry, Japan, 2002). It has furthermore been reported to exert estrogenic potential in a yeast proliferation assay (Zacharewski *et al.*, 1998), a reporter gene assay (Zacharewski *et al.*, 1998), and cell growth studies using human breast cancer cells (Komer *et al.*, 1998; Jones *et al.*, 1998). However, no change in uterine weight was found in a uterotrophic assay (Coldham *et al.*, 1997; Ministry of Economy, Trade and Industry, Japan, 2002) and Hershberger assay results showed reduced weight in the bulbospongiosus muscle plus levator ani muscle, pointing to an anti-androgenic effect (Ministry of Economy, Trade and Industry, Japan, 2002). BBP has in fact been reported to induce reproductive toxicity, as evidenced by pre- or postimplantation loss in rats given the compound during early pregnancy (Ema *et al.*, 1998) as well as decreased anogenital distance (AGD), testis or epididymal weights, increase in the incidence of reproductive organ malformations, retention of nipples and areolae or delayed preputial separation of the penis in the male offspring in the rats when administered during late pregnancy (Ema and Miyawaki 2002), during the prenatal stage (Gray *et al.*, 2000), during gestation and lactation (Ashby *et al.*, 1997) or in two-generation reproductive studies (Nagao *et al.*, 2000; Tyl *et al.*, 2004).

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We have performed a two-generation reproductive toxicity study with extra parameters to confirm the utility of this approach for identifying non-steroid chemicals with endocrine activity by focusing on reproductive capacity in parental animals and on growth and development in offspring.

MATERIALS AND METHODS

Chemicals

Butyl benzyl phthalate (BBP) (Lot number G1o1, purity: 98.0%) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Stability of the preparation was confirmed by analyses prior to the beginning and at the end of the study.

Animals and housing conditions

Male and female Crj:CD®(SD)IGS rats were purchased from Charles River Japan, Inc (Yokohama, Japan). After quarantine and acclimation, only healthy animals showing favorable body weight gain and a good general condition were used for the study. Groups of twenty-four males and females were allocated to each treatment using body weight-stratified randomization.

The animals were placed individually in stainless steel cages with metal net floors (280W × 440D × 150H mm, Tokiwa Scientific Equipment Co., Tokyo, Japan) in a breeding room with a barrier system under control conditions of temperature (21-25°C), relative humidity (40-70%), ventilation (10-15 times/hr), and 12-hr light and darkness cycling (lights on at seven o'clock and off at 19 o'clock). During the period from gestation day 17 (GD17) to weaning day, the animals were bred individually or with individual littermates in polycarbonate cages with flat floors (265W × 426D × 200H mm, Tokiwa Scientific Equipment Co.) and standard bedding (Sun-flake, Charles River Japan, Inc.). They were allowed free access to solid feed (NIH-07M, sterilized with 30kGy gamma rays, CLEA Japan, Inc., Tokyo, Japan), and to Hita-city public water (chlorine-added) using automatic water-suppliers or bottles.

Dosing

The doses of the test substance were selected, based on the results of a preliminary two-generation reproductive toxicity study of BBP in rats (executed by Chemicals Evaluation and Research Institute, Tokyo, Japan). In the preliminary study, BBP was administered orally by gavage at the doses of 125, 250, 500, and 1,000 mg/kg/day to eight Crj:CD®(SD)IGS rats of each sex per group for four weeks before mating for 10 weeks and during the mating period, and after that until weaning of the offspring in the females, and after completion of mating until one day prior to the day of autopsy in the males. The effects on reproductive capacity in the parental animals and the growth in the offspring were investigated. As a result, the dose of 1,000 mg/kg caused inhibition of dams' body weight gain and stillbirth or death of all offspring of all dams. Although stillborn or dead pups were found at a dose of 500 mg/kg, slight decrease in ovarian weight was the only observation at the doses less than 250 mg/kg. From these findings, in this study, 400 mg/kg/day was selected as the highest dose for the present study, at which some effects on the delivered pups was anticipated, with middle and low doses of 200 and 100 mg/kg/day, respectively, with division by a common ration of 2.

The compound administration was started at five weeks old in the F0 parents and when the animals were three weeks old in the F1 parents. The test substance was dissolved in olive oil (Fujimi Pharmaceutical Co., Osaka, Japan, Lot number 016OAA), and administered orally by gavage before mating for 10 weeks and then during the mating period (males and females were returned to their cages each morning) in both sexes; then during the gestation period, delivery days (administered before delivery on that day or after delivery for the cases just giving birth) and during the lactation period in females; and in the period up to autopsy after the delivery completion of offspring in the males. Each animal received 5 mL/kg for seven days per week containing doses of 0, 100, 200, and 400 mg/kg/day. At the administration, a disposable catheter (Terumo Co., Tokyo, Japan, polyvinyl chloride urethral catheter, external diameter 8/3 mm) was used to connect with an injection syringe for the females at five weeks old or older and the males 5-9 weeks old, and a disposable catheter (Terumo Co., polyvinyl chloride urethral catheter, external diameter 10/3 mm) for the males 10 weeks old or older. For the males and females after weaning to four weeks old, a metal gastric tube was connected with an injection syringe for test substance administration.

Observations

1. Parental animals

1) Clinical signs

All animals were observed for their clinical signs once or more daily during the period from the start of the administration until one day prior to autopsy after weaning (postnatal day (PND) 21) of the delivered pups in the females, including the nursing conditions of the F1 pups, and during the period until the one day prior to autopsy in the males.

2) Body weights and body weight gain, food consumption and feeding efficiency

The body weights of males were measured on the start day of administration and subsequently once a week until autopsy as a rule. The body weights of females was measured on the start day of administration and subsequently once a week, and after gestation, on GDs 0, 7, 14, and 20, and on PNDs 0, 4, 7, 14, and 21. Body weight gain in males was calculated from the values during the period from the start of the administration up to week 14. The gain in females was calculated from the values during the period from the start of the administration up to week 10 of administration for the pre-breeding period, up to GD 20 for the gestation period, and up to PND 21 for the lactation period. Food consumption was measured at the same time as body weights, or supplied and remaining food amounts were measured for each cage whenever the food was supplied. The mean food consumption value per rat was calculated by dividing by the number of food-supply days. In the females during the lactation period, the values were calculated as the food consumption per litter. However, measurement of food consumption was not performed during the mating period. Feeding efficiency was calculated, based on the body weight values on the start day of administration for the males, and on the start day of administration, GD 0, and PND 0 for the females.

3) Confirmation of mating, pregnancy and selection of parental animals

For the F0 parents, when both the males and females became 15 weeks old, females were moved to male cages in the evening to mate using a continuous cohabiting system at a sex ratio of 1:1. For the F1 parents, when the males and females became 13 weeks old, females were similarly moved to male cages. From the following day, the existence of a vaginal plug or sperm in the vaginal smear was investigated daily in the morning. When a vaginal plug or sperm in the vaginal smear was found, it was judged that the mating had been conducted, and that day was determined as GD 0, and the pregnant females were moved to a special cages to breed separately. The females for which the mating was not confirmed were returned to the original cages, and the mating was attempted repeatedly for a maximum two weeks or until mating was confirmed by the above-described method. Pregnancy was confirmed by delivery or the finding of an implantation scar when the uterus was cut along the long axis and soaked in ammonium sulfide aqueous solution. The F1 parental animals were selected from each litter of the F0 animals delivered over five days, including the day when most deliveries were found in all groups, one or two male and female offspring in each cage being selected randomly at the time of weaning.

4) Reproductive capacity

Based on the observations during the reproductive period in the parental animals, the following indices were calculated. As criteria for sexual maturation, the females were examined for vaginal opening on PND 36 and the males for preputial separation of the penis on PND 43. The females were examined for their estrous cyclicity using vaginal smears for 2 weeks before mating. After mating was performed, the mating index, fertility index, gestation index, number of days required for copulation, gestation length, number of implantations, delivery index and number of the pups delivered were assessed.

5) Sperm examinations

Sperm examinations were conducted for 10 males mated with the females delivering the F1 pups in each group at autopsy. The numbers of sperm in the testis and the caudal epididymis were counted under a microscope using a hemocytometer. Epididymal sperm mortility was investigated using a Hamilton sperm mortility analyzer (HTM-IVOS; Hamilton Thorne Research, Inc., Beverly, MA, USA). Smear-stained specimens were prepared and epididymal sperm morphology was observed for 200 individual sperm under a microscope and the rate for morphologically normal sperm was determined.

6) Measurement of hormone levels

On the day of autopsy for the males and on the day of autopsy when the estrous cycle indicated the estrous stage after weaning of the F1 pups for the females, blood was collected from six males and females in each group from the abdominal aorta after ether anesthesia under non-fasting conditions, and serum was obtained. The serum LH, FSH, and testosterone concentrations were measured by EIA methods. Estradiol concentrations were measured by an RIA method at Panapharm Laboratories Co., Ltd

(Kumamoto, Japan). As no abnormalities were detected in levels of any hormone measured for animals receiving 400 mg/kg, measurement for the doses of 100 and 200 mg/kg was omitted except for FSH in the males.

7) Organ weights

In the F0 and F1 parental animals, wet weights were measured for the liver, kidneys, testes, epididymis, ventral prostate, seminal vesicles (including the coagulating glands), ovaries uterus, brain (cerebrum, cerebellum), spleen, pituitary gland, thyroid (including the parathyroid), and adrenal glands. With the bilateral organs, right and left were measured separately. For the prostate, only the ventral lobe was weighted. The thyroid and parathyroid, and seminal vesicles (including the coagulating glands) were fixed in 10% neutral buffered formalin solution for measurement on the following day.

8) Autopsy

On the day when the females showed the estrous stage on observation of the estrous cycle after weaning of the F1 pups, and on the day of autopsy for the males, they were sacrificed under anesthesia, and were underwent a macroscopic examination of all organs and tissues. Dead animals or animals in a moribund conditions, and the dams whose delivered pups all died were autopsied as early as they were found. As with surviving animals, the tissues were collected, organs were weighed, and histopathological examinations were conducted.

9) Histopathological examinations

In the F0 and F1 parents, histopathological examinations were conducted for the liver, heart, kidneys, testes, epididymis, prostate, seminal vesicles, ovaries, uterus, vagina, brain, spleen, thymus, pituitary gland, thyroid (including the parathyroid), adrenal glands, and mammary glands resected and routinely processed for paraffin-embedding and sectioning.

2. Offspring

1) Indices related to the offspring

External surfaces and the sex ratios of the delivered live pups were examined on PND 0. The animals were observed for clinical signs everyday during the lactation period. On PND 4, the anogenital distance (AGD) was measured, and then the number of the pups in each litter was adjusted randomly to eight (four males and four females where possible) and the pups not selected were sacrificed under ether anesthesia, and stored in 10% neutral buffered formalin solution after examination of the external surfaces. Body weights were measured individually on PNDs 0, 4, 7, 14, and 21 and viability was assessed on PND 0, PND 4, and PND 21 (weaning rate).

2) Physical development

In all live pups in each litter, the existence in the following items was recorded: pinna unfolding on PND 4, incisor eruption on PND 13, eye opening on PND 15, vaginal opening on PND 36, and preputial separation of the penis on PND 43.

3) Response test

The existence of the following items was recorded for all live pups in each litter: systemic pain response on PND 5, negative geotaxis on PND 9, and air righting reflex and pinna reflex on PND 16.

4) Organ weighs

The wet weights of the brain, spleen, and thymus were measured for the F1 and F2 offspring of one animal in each litter undergoing autopsy on the day of weaning (PND 21).

5) Autopsy

The F1 offspring not selected as parental animals on PNDs 25 - 27, and the F2 offspring on PND 21 were sacrificed under anesthesia, and macroscopically examined. Dead animals or animals in a moribund condition were autopsied as early as they were found.

Statistical analyses

The Bartlett's analysis for homogeneity of variance (Bartlett, 1937) was conducted for the body weights, body weight gain, food consumption, feeding efficiency, the organ weights, the number of days required for mating, gestation length, the number of implantations (scars), sperm examination items (the number of sperm), and hormone measurements in the F0 and F1 generations, the body weights in the F2 generation, and the number of pups delivered, the number of the delivered live pups, AGD, and the organ weights of the F1 and F2 pups. When variance was uniform at the 5% level of significance, one-way ANOVA was conducted. If a significant difference was detected, the Dunnett's multiple comparison test (Dunnett, 1964) was applied for detection of differences between control and treatment groups. When the variance was not uniform, the Kruskal-Wallis's rank sum test (Kruskal and Wallis, 1952) was conducted. Then if a significant difference was detected, the non-parametric Dunnett method was applied for analysis of significant differences. The Chi-square test (Snedecor and Cochran, 1967) was employed for analyses of the frequency of clinical signs, gestation index, delivery index, rate of abnormal estrous cycling, and the frequency of autopsy or histopathological findings in the F0 and F1 generations, the sex ratio, physical developmental items, and response test items in the F1 and F2 pups, and the mating, fertility and gestation indices in the F0 and F1 generations. However, when the secondary degree was 10 or less, the Fisher's exact test (Fisher, 1950) was conducted. The Kruskal-Wallis test was conducted for analyses of sperm examination items (sperm motility and morphology), and feeding efficiency in the F0 and F1 generations, and rate for abnormalities of external surfaces, viability on PND 0 and PND4, and weaning rates in the F1 and F2 pups. When a significant difference was detected, testing by the non-parametric Dunnett method was conducted. The following indices were treated with each litter as specimen units; abnormalities rates of the external surfaces, viability on PND 0 and PND4, weaning rates, and body weight values during the period from the day of birth up to weaning in the F1 and F2 pups.

RESULTS

Parental animals

1. Clinical signs

Temporally salivation immediately after the administration was observed in the F0 and F1 animals throughout the administration period in both sexes of the 0, 100, 200, and 400 mg/kg groups, and the frequency was increased significantly in F0 males of the 400 mg/kg group, and at more than 100 mg/kg F1 males and F0 and F1 females receiving 200 mg/kg. One F1 female in the 200 mg/kg group showed reduced locomotor activity, a reduced respiratory rate, reddish tears, staining around the nose, mouth and lower abdomen and nasal bleeding, and eventually died.

2. Body weights and body weight gain

No abnormalities in body weights or body weight gain due to BBP were found in either sex of any dose group in the F0 and F1 parents (Figs. 1 and 2).

3. Food consumption and feeding efficiency

1) F0 animals

In males, significant increase in the food consumption was found at week 10 of administration in the 200 mg/kg group and at weeks 2 and 3 of administration in the 400 mg/kg group. In females, the feeding efficiency was decreased significantly on GDs 0-7 in the 400 mg/kg group. With 200 mg/kg, elevated values for food consumption were observed at weeks 6-10 of administration, together with significantly higher levels of feeding efficiency at weeks 0-2, 0-4 and 0-10.

2) F1 animals

In the males, the feeding efficiency in the 400 mg/kg group was decreased significantly at weeks 0-5 and weeks 0-10. In the females, this was also the case at weeks of 0-7 and weeks 0-10, but significant increase on PNDs 0-4 and PNDs 0-21 were found. Other observations included significantly increased food consumption at week 10 in the 100 mg/kg group, lowered feeding efficiency on GDs 0-20 in the 200 mg/kg group, and increased food consumption at week 10 in the 400 mg/kg group.

4. Reproductive capacity (including sperm parameters)

Among indices related to the reproductive capacity of the parents, no abnormalities in the following items were found in either F0 or F1 parents in any dose group: estrous cyclicity, mating index, number of days required for mating, gestation length, number of implantations, delivery index, gestation index, number of the pups delivered, number of sperm in the testis and epididymis, and epididymal sperm motility and morphology (morphologically abnormal sperm rate). With regard to the fertility index, no abnormality was found in F0 parents, but the results for F1 parents showed low values including in the control. In addition, a significantly low rate for completed preputial separation of the penis was evident in the 400 mg/kg group of F1 parents (Tables 1 and 2).

5. Serum hormones

No significant differences were apparent between the 400 mg/kg and control groups regarding serum FSH, LH, testosterone and estradiol concentrations in the F0 and F1 parents (Table 3).

6. Organ weights

1) F0 animals

In the groups receiving 200 mg/kg or more, significantly elevated values were found for absolute renal weights in the males, and the absolute and relative hepatic weights and absolute renal weights in females. In the 400 mg/kg group, significantly high relative hepatic and renal weights and low absolute epididymal weights in males, and high relative renal weights in females were also apparent. Reduced relative uterine weights in females of the 200 mg/kg group was another change (Tables 4 and 5).

2) F1 animals

Significantly lowered absolute epididymal and elevated relative hepatic weights in males given 200 mg/kg or more, significantly lowered absolute weights of seminal vesicles and elevated relative weights of thyroid in males and increased relative hepatic weights in females of the 400 mg/kg group were notedTables 6 and 7).

7. Autopsy and histopathological examinations

1) F0 animals

No abnormal autopsy findings were observed in either sex in any dose group. Histopathological findings in males of the 400 mg/kg group were increased hyperplasia of Leydig cells of the testes and decreased spermatozoa in the lumina of the epididymes.

2) F1 animals

On autopsy, significant increase in frequency of small testes was found in the males of the 400 mg/kg group. Softening of the testes was observed with 100 mg/kg or more, and aplasia and/or dysplasia and small size of the epididymes at 400 mg/kg. As histopathological findings, increase in the frequency of diffuse atrophy of the seminiferous tubules and hyperplasia of Leydig cells in the testes (Photo 1) was observed significant in the 400 mg/kg males. Diffuse atrophy of testicular seminiferous tubules, decreased spermatozoa and residual germ cells in epididymal lumina (Photo 2) were all found in the groups receiving 100 mg/kg or more. Bilateral or unilateral partial aplasia, or unilateral aplasia of the epididymes was also observed in the 400 mg/kg group (Tables 8 and 9).

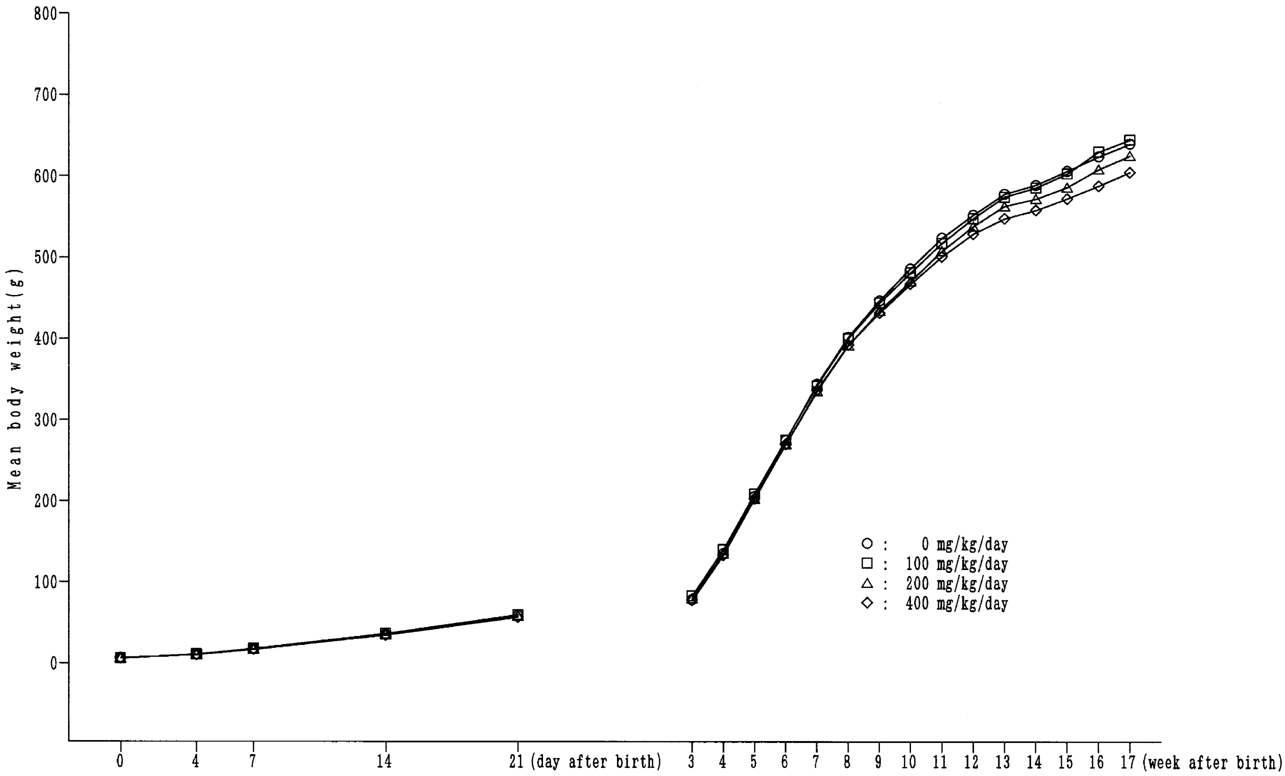

Fig. 1. Body weight of F1 male rats exposed to BBP.

Offspring

1. Clinical signs

No abnormalities in clinical signs due to BBP including viability were found in either sex of any dose group in the F1 and F2 offspring.

2. Body weights

Significant lowered body weights on PND 0 were evident in F1 male offspring of the groups receiving 100 mg/kg or more and in the F2 male and female offspring in the 100 and 400 mg/kg groups. No abnormalities were found in the F1 female offspring (Figs. 1, 2, 3 and 4).

3. Indices related to offspring (including physical development)

No abnormalities were found in the sex ratio of the delivered pups, and external surfaces in the F1 and F2 offspring. Pinna unfolding, incisor eruption, and eye opening were normal in both generations with all doses.

1) F1 offspring

An increase in the AGD (mm/cubic root of body weight) was observed in the female pups of the groups receiving 100 mg/kg or more (Table 10).

2) F2 offspring

In the males, decrease in the AGD (mm/cubic root of body weight) was found in the groups given 100 mg/kg or more, along with decrease in absolute values for AGD (mm) in 100 and 400 mg/kg groups(Table 11).

4. Response test

No abnormalities in indices for the systemic pain response, negative geotaxis, air righting reflex, and pinna reflex were found in F1 and F2 offspring of either sex in any dose group.

5. Autopsy findings

Autopsy of F1 and F2 pups which died during the lactation period, F1 weanlings not selected as F1 parents, and F2 weanlings, revealed no changes attributable to BBP in any of the dose groups.

6. Organ weights

The absolute and relative splenic weights in

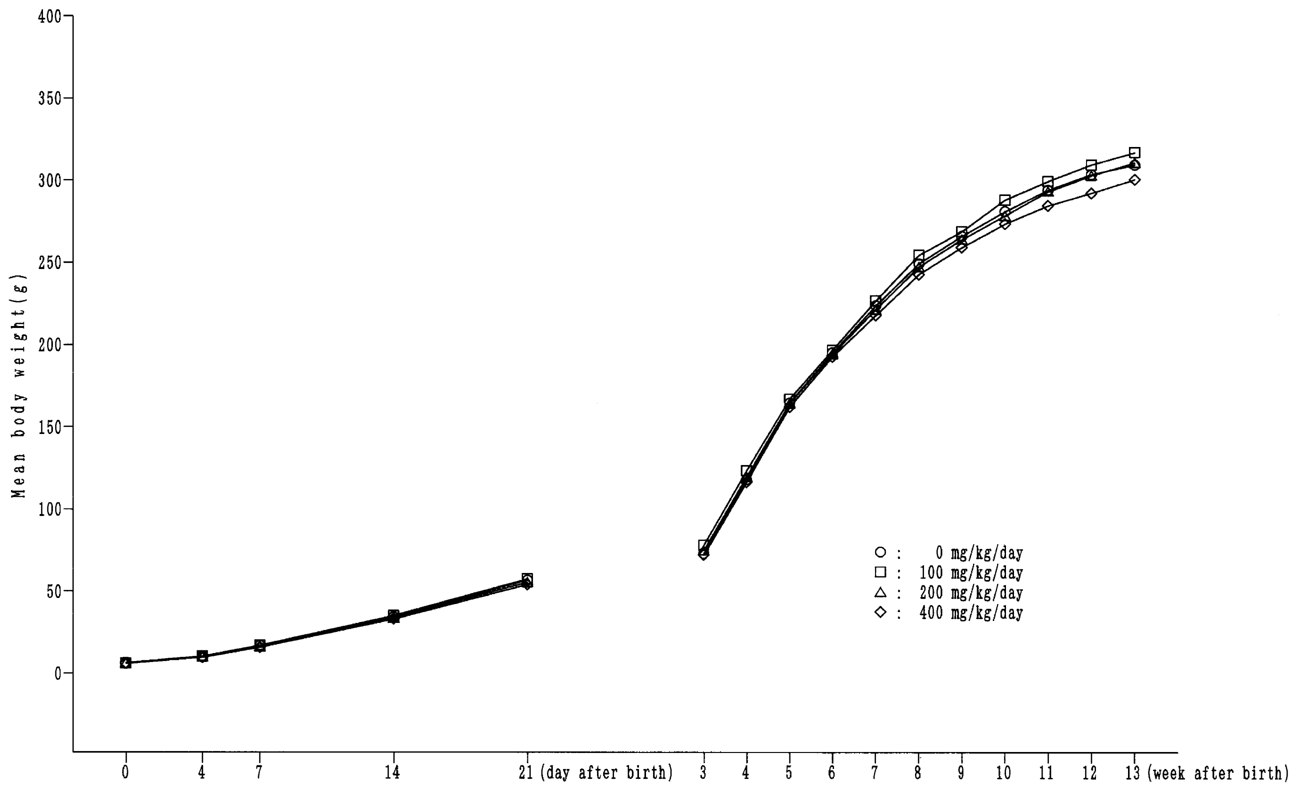

Fig. 2. Body weight of F1 female rats exposed to BBP.

males of the 400 mg/kg group demonstrated low values in the F1 and F2 offspring (Tables 12 and 13).

DISCUSSION

In the present study, salivation was observed in both sexes of all dose groups in the F0 and F1 parents. Since no other apparent effects were found on the auto-

Table 1. Reproductive capacity of F0 parents exposed toBBP.

Sex		BBP(mg/kg/day) 0	100	200	400
Male	Mating index	24/24 (100)	22/24 (91.7)	22/23 (95.7)	23/24 (95.8)
	Fertility index	20/24 (83.3)	19/22 (86.4)	20/22 (90.9)	21/23 (91.3)
	Number of sperm in testes ($\times 10^6$/g)	93.0 ± 15.3	99.0 ± 12.2	85.1 ± 17.0	91.3 ± 7.5
	Number of sperm in caudal epididymes ($\times 10^6$/g)	611.7 ± 112.4	666.4 ± 66.6	621.8 ± 51.0	616.0 ± 72.0
	Epididymal sperm motility (%)	70.8 ± 18.7	64.6 ± 21.4	73.6 ± 17.7	58.3 ± 15.5
	Epididymal sperm abnormality (%)	0.3 ± 0.4	– [a]	–	0.2 ± 0.2
Female	Normal estrous cyclicity	24/24 (100)	24/24 (100)	21/23 (91.3)	22/24 (91.7)
	Mating index	24/24 (100)	22/24 (91.7)	22/23 (95.7)	23/24 (95.8)
	Fertility index	20/24 (83.3)	19/22 (86.4)	20/22 (90.9)	21/23 (91.3)
	Gestation index	20/20 (100)	19/19 (100)	20/20 (100)	21/21 (100)
	Number of days required for copulation	3.5 ± 2.5	2.0 ± 1.2	2.3 ± 1.3	3.6 ± 2.7
	Gestation length (day)	21.9 ± 0.37	21.7 ± 0.45	21.9 ± 0.31	21.9 ± 0.36
	Number of implantations	14.9 ± 1.80	12.9 ± 3.14	14.0 ± 3.52	14.0 ± 2.16
	Delivery index	259/298 (86.9)	232/245 (94.7)	265/280 (94.6)	273/293 (93.2)
	Number of pups delivered	12.8 ± 3.32	12.1 ± 3.18	13.1 ± 3.71	12.8 ± 2.19

Values are mean ± SD.
Values in parentheses are percentages.
[a] Not examined.

Table 2. Reproductive capacity of F1 parents exposed toBBP.

Sex		BBP(mg/kg/day) 0	100	200	400
Male	Preputial separation of penis	23/24 (95.8)	17/24(70.8)	22/24(91.7)	14/24 (58.3)**
	Mating index	21/23 (91.3)	22/24(91.7)	20/24(83.3)	20/24 (83.3)
	Fertility index	16/21 (76.2)	21/22(95.5)	17/20(85.0)	13/20 (65.0)
	Number of sperm in testes ($\times 10^6$/g)	102.8 ± 8.7	108.8 ± 12.7	103.4 ± 13.5	100.3 ± 11.0
	Number of sperm in caudal epididymes ($\times 10^6$/g)	619.8 ± 62.8	637.7 ± 89.6	663.0 ± 93.0	612.1 ± 95.9
	Epididymal sperm motility (%)	56.3 ± 16.7	71.2 ± 20.2	60.0 ± 21.2	66.1 ± 12.8
	Epididymal sperm abnormality (%)	0.0 ± 0.0	– [a]	–	0.1 ± 0.2
Female	Vaginal opening	24/24 (100)	24/24 (100)	22/24 (91.7)	22/24 (91.7)
	Normal estrous cyclicity	21/23 (91.3)	17/24 (70.8)	23/24 (95.8)	24/24 (100)
	Mating index	21/23 (91.3)	22/24 (91.7)	20/24 (83.3)	20/24 (83.3)
	Fertility index	16/21 (76.2)	21/22 (95.5)	17/20 (85.0)	13/20 (65.0)
	Gestation index	15/16 (93.8)	20/21 (95.2)	16/17 (94.1)	13/13 (100)
	Number of days required for copulation	3.0 ± 1.8	3.4 ± 3.2	3.1 ± 2.1	3.1 ± 1.9
	Gestation length (day)	22.1 ± 0.26	21.8 ± 0.55	22.0 ± 0.52	22.1 ± 0.49
	Number of implantations	13.3 ± 3.77	14.1 ± 4.67	10.8 ± 4.68	12.8 ± 3.56
	Delivery index	184/213 (86.4)	276/297 (92.9)	163/183 (89.1)	151/166 (91.0)
	Number of pups delivered	12.3 ± 2.84	13.8 ± 3.69	10.2 ± 4.37	11.6 ± 3.40

Values are mean ± SD.
Values in parentheses are percentages.
[a] Not examined.
** Significantly different from the controls at $p<0.01$.

nomic nervous system and the frequency was increased in a dose-dependent manner, this was considered to be caused by BBP irritation (Mallette and von Haam, 1952; Hammond *et al*., 1987). However, because the salivation was only transiently observed immediately after administration and no other change was recognized, it was considered to have limited toxicological significance.

No abnormalities for body weights and body weight gain by BBP were found in both sexes of any dose group in parents.

Regarding feeding efficiency, significant decrease in the F0 females and both F1 sexes given 400 mg/kg group together with altered food consumption or feeding efficiency. However, they were all transient not consistently observed across generation and without dose dependence. Thus, they were considered to be incidental or that have little toxicological significance.

Among indices related to the reproductive capacity of parents, pre- or postimplantation loss has been reported (Ema and Miyawaki, 2002), and although no abnormality including the number of pups delivered was here found in the F0 parents, the results for the F1 parents showed lowering of the fertility index. Because decreased spermatozoa were found in the groups receiving 100 mg/kg or more, effects of BBP were unequivocal. However, the control values were low, so that significance was not achieved for many parameters. While the changes in testes and epididymes observed in males getting females pregnant and the other males belonging to the non-pregnant pairs including other dose groups were compensative unilateral, those observed in three males from non-pregnant pairs in of 400 mg/kg group were bilateral and sufficiently to assume they were the cause of failure to fertilize. In males of the same dose group, because the findings included small testis size (autopsy finding), significant increase in the appearance diffuse atrophy of the seminiferous tubules and/or Leydig cell hyperplasia in the testis (histopathological findings) in line with the lower fertility index reported for an earlier in study with BBP (Piersma *et al*., 1995), it was very likely considered that the disorder of the testis and epididymis induced by BBP in the males might cause to decrease the fertility index. In the 400 mg/kg group of F1 parents, significant lowering of the proportion of males with completed preputial separation of the penis was here apparent, again with the delay described previously with BBP (Ashby *et al*., 1997, Nagao *et al*., 2000). However, the direct reason for low reproductive results was not clarified and despite pathological changes observed in the reproductive organs of the males, no effects on sperm parameters or hormone levels were found. From the available data, although a conclusion could not been drawn in terms of lack of effects of BBP on reproductive capacity at 200 mg/kg, it can be considered unlikely that this dose causes reproductive toxicity.

As already noted, no significant difference was here found between the 400 mg/kg and control groups for any serum hormones measured, including testosterone, in either sex of F0 and F1 parents. Although it has

Table 3. Hormone measurement for F0 parents exposed to BBP.

Sex		BBP (mg/kg/day) 0	100	200	400
Male	No. of animals examined	6	6	6	6
	LH (ng/ml)	18.8 ± 4.9	– [a]	–	14.3 ± 2.7
	FSH (ng/ml)	104.8 ± 29.2	86.4 ± 17.9	134.8 ± 43.5	79.2 ± 8.1
	Testosterone (pg/ml)	6353 ± 3389	–	–	3706 ± 1636
	Estradiol (pg/ml)	5.1 ± 1.6	–	–	4.9 ± 2.5
Female	No. of animals examined	6	0	0	6
	LH (ng/ml)	8.5 ± 1.5	– [a]	–	8.7 ± 1.9
	FSH (ng/ml)	68.9 ± 7.5	–	–	57.7 ± 10.3
	Testosterone (pg/ml)	27 ± 5	–	–	27 ± 4
	Estradiol (pg/ml)	7.0 ± 3.8	–	–	6.4 ± 2.1

Values are mean ± SD.
[a] Not examined.

been reported that BBP can increase serum testosterone and reduced the FSH concentration (Clemens *et al*., 1978; Nagao *et al*., 2000), such changes were not present in this study. Because these were only observed at a dose of 500 mg/kg in the previous reports and the maximum dose used in this study was 400 mg/kg, the lack in the present case might be attributed to dose differences.

Effects of BBP on parents included significantly increase in renal weights in both sexes and hepatic weights in females in the groups given 200 mg/kg or more, as well as lowering of epididymal weights at 400 mg/kg. Although relative uterine weights were found to be reduced in F0 females of the 200 mg/kg group, it seems to be unlikely that the change was caused by BBP, because it was not dose-dependent and was not

Table 4. Organ weights for F0 male parents exposed to BBP.

			BBP (mg/kg/day)			
			0	100	200	400
No. of animals examined			20	19	20	21
Terminal weight (g)			580.3 ± 33.2	590.5 ± 50.9	602.2 ± 63.6	575.0 ± 56.0
Liver		(g) [a]	20.46 ± 1.34	21.53 ± 3.18	22.36 ± 3.38	23.33 ± 3.70
		(g/100 g) [b]	3.53 ± 0.18	3.63 ± 0.29	3.71 ± 0.33	4.04 ± 0.32**
Kidney	right	(g)	1.79 ± 0.17	1.87 ± 0.17	1.91 ± 0.21	1.94 ± 0.21(20)
		(g/100 g)	0.31 ± 0.03	0.32 ± 0.03	0.32 ± 0.03	0.34 ± 0.04(20)
	left	(g)	1.74 ± 0.15	1.84 ± 0.18	1.88 ± 0.23*	1.90 ± 0.18(20)*
		(g/100 g)	0.30 ± 0.03	0.31 ± 0.03	0.32 ± 0.03	0.33 ± 0.03(20)**
Testis	right	(g)	1.71 ± 0.13	1.80 ± 0.13	1.78 ± 0.17	1.69 ± 0.18
		(g/100 g)	0.30 ± 0.03	0.31 ± 0.03	0.30 ± 0.04	0.30 ± 0.03
	left	(g)	1.72 ± 0.14	1.78 ± 0.13	1.79 ± 0.17	1.68 ± 0.19
		(g/100 g)	0.30 ± 0.03	0.30 ± 0.03	0.30 ± 0.04	0.29 ± 0.03
Epididymis	right	(g)	0.66 ± 0.07	0.68 ± 0.05	0.67 ± 0.05	0.63 ± 0.07
		(g/100 g)	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
	left	(g)	0.65 ± 0.07	0.66 ± 0.06	0.66 ± 0.05	0.61 ± 0.06*
		(g/100 g)	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Ventral prostate		(g)	0.77 ± 0.21	0.67 ± 0.20	0.74 ± 0.17	0.63 ± 0.18
		(g/100 g)	0.13 ± 0.04	0.11 ± 0.04	0.12 ± 0.03	0.11 ± 0.03
Seminal vesicle		(g)	1.88 ± 0.20	1.85 ± 0.18	1.88 ± 0.20	1.74 ± 0.24
		(g/100 g)	0.33 ± 0.04	0.32 ± 0.04	0.32 ± 0.04	0.30 ± 0.04
Brain		(g)	2.09 ± 0.06	2.12 ± 0.07	2.10 ± 0.07	2.09 ± 0.09
		(g/100 g)	0.36 ± 0.02	0.36 ± 0.03	0.35 ± 0.03	0.37 ± 0.03
Spleen		(g)	0.83 ± 0.11	0.77 ± 0.10	0.79 ± 0.11	0.80 ± 0.16
		(g/100 g)	0.14 ± 0.02	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.02
Pituitary		(mg)	12.9 ± 1.4	13.0 ± 1.2	13.4 ± 1.3	13.1 ± 1.8
		(mg/100 g)	2.2 ± 0.3	2.2 ± 0.2	2.2 ± 0.2	2.3 ± 0.3
Thyroid		(mg)	24.0 ± 4.7	22.5 ± 4.0	25.3 ± 4.4	26.1 ± 4.1
		(mg/100 g)	4.2 ± 0.8	3.8 ± 0.6	4.2 ± 0.7	4.6 ± 1.0
Adrenal gland	right	(mg)	27.6 ± 4.3	28.4 ± 2.5	28.6 ± 4.4	28.8 ± 4.2
		(mg/100 g)	4.7 ± 0.6	4.8 ± 0.6	4.8 ± 0.7	5.0 ± 0.8
	left	(mg)	29.9 ± 5.4	29.6 ± 2.7	31.5 ± 4.6	29.4 ± 4.3
		(mg/100 g)	5.1 ± 0.8	5.0 ± 0.6	5.2 ± 0.7	5.1 ± 0.8

Values are mean ± SD.
Values in parenteses are the numbes of animals examined.
[a] Absolute weight.
[b] Relative weight.
* Significantly different from the controls at $p<0.05$.
** Significantly different from the controls at $p<0.01$.

seen in the F1 parents. It has been reported that BBP causes decrease in weights of the testis, epididymis and seminal vesicle and increases in the liver and kidney (Ashby *et al*., 1997; Nagao *et al*., 2000), and most of these findings were reproduced in our study. Regarding reduction in the weights of testes (Piersma *et al*., 1995; Gray *et al*., 2000) or male genital organs including the testes (Tyl *et al*., 2004) reported in previous studies treated with BBP was not observed in this study, the effective doses for the weight reduction of these organs were all more than 750 mg/kg (11,250 ppm in diet, approximately 750 mg/kg/day, for Tyl's report), and, thus, the lack in the present case might be attributed to dose differences. Significant increase in relative thyroidal weights seen in the F1 males of 400 mg/kg group was considered to be incidental, since no significant difference in its absolute weights or no abnormalities were found in the F0 parents. Increase in hepatic and renal weights in association with testicular changes found in groups receiving 200 mg/kg or more have also been observed for other phthalate esters (Oishi and Hiraga, 1980; Lake *et al*., 1982; NTP, 1993; Wine *et al*., 1997), and therefore, the change was not considered to be specific for BBP. It is suggested that the disrupting effects of phthalate esters on the endocrine system may be associated with reactions with lipid metabolism mediated by the peroxisome proliferator-activated receptor (PPAR) (Bojes and Thurman, 1996;

Table 5. Organ weights for F0 female parents exposed to BBP.

			BBP (mg/kg/day)			
			0	100	200	400
No. of animals examined			20	19	19	20
Terminal weight (g)			303.6 ± 25.4	304.1 ± 21.6	320.8 ± 17.6	305.3 ± 21.2
Liver		(g) [a]	11.36 ± 1.34	11.71 ± 0.75	12.70 ± 0.91**	12.56 ± 1.10**
		(g/100 g) [b]	3.74 ± 0.24	3.86 ± 0.16	3.96 ± 0.27*	4.12 ± 0.26**
Kidney	right	(g)	1.06 ± 0.13	1.09 ± 0.08	1.18 ± 0.10**	1.14 ± 0.11*
		(g/100 g)	0.35 ± 0.03	0.36 ± 0.03	0.37 ± 0.03	0.37 ± 0.03*
	left	(g)	1.01 ± 0.12	1.18 ± 0.10	1.13 ± 0.11**	1.13 ± 0.12**
		(g/100 g)	0.33 ± 0.03	0.35 ± 0.02	0.35 ± 0.03	0.37 ± 0.03**
Ovary	right	(g)	50.1 ± 10.4	48.0 ± 6.7	50.7 ± 9.4	46.6 ± 7.4
		(g/100 g)	16.4 ± 2.5	15.7 ± 1.7	15.8 ± 3.0	15.3 ± 2.3
	left	(g)	48.1 ± 9.0	47.9 ± 5.8	46.7 ± 9.9	46.3 ± 8.3
		(g/100 g)	15.9 ± 2.9	15.8 ± 2.1	14.6 ± 3.1	15.2 ± 2.7
Uterus		(g)	0.79 ± 0.14	0.73 ± 0.12	0.72 ± 0.11	0.76 ± 0.10
		(g/100 g)	0.26 ± 0.04	0.24 ± 0.04	0.22 ± 0.03**	0.25 ± 0.03
Brain		(g)	1.88 ± 0.06	1.86 ± 0.07	1.94 ± 0.08	1.89 ± 0.08
		(g/100 g)	0.62 ± 0.06	0.62 ± 0.06	0.61 ± 0.04	0.62 ± 0.05
Spleen		(g)	0.50 ± 0.06	0.49 ± 0.05	0.53 ± 0.06	0.49 ± 0.07
		(g/100 g)	0.17 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.16 ± 0.02
Pituitary		(mg)	15.7 ± 2.7	14.7 ± 2.2	15.7 ± 2.1	14.1 ± 2.0
		(mg/100 g)	5.2 ± 0.9	4.8 ± 0.6	4.9 ± 0.7	4.6 ± 0.6
Thyroid		(mg)	20.2 ± 4.7	19.3 ± 4.6	18.9 ± 3.6	22.2 ± 6.1
		(mg/100 g)	6.7 ± 1.6	6.4 ± 1.5	5.9 ± 1.1	7.3 ± 1.9
Adrenal gland	right	(mg)	32.1 ± 4.4	32.7 ± 4.8	33.3 ± 5.4	31.4 ± 5.0
		(mg/100 g)	10.6 ± 1.2	10.8 ± 1.5	10.4 ± 1.8	10.3 ± 1.7
	left	(mg)	34.8 ± 6.1	35.9 ± 4.8	36.2 ± 5.5	33.5 ± 4.7
		(mg/100 g)	11.5 ± 1.9	11.8 ± 1.6	11.3 ± 1.9	11.0 ± 1.6

Values are mean ± SD.
[a] Absolute weight.
[b] Relative weight.
* Significantly different from the controls at p<0.05.
** Significantly different from the controls at p<0.01.

Ward *et al*., 1998; Willhite, 2001). The possibility that BBP also impacts on these organs by similar mechanisms can be speculated.

Although hyperplasia of Leydig cells of the testes and decreased spermatozoa in the lumina of the epididymis observed in the F0 400 mg/kg group were slight and occurred spontaneously, they were also observed in the F1 parents so that it could not be denied the effects were related to BBP. In the F1 parents, softening of the testes in the groups receiving 100 mg/kg or more, a significant increase in the frequency of small testes size and aplasia and/or dysplasia, and small size of the epididymis in the group of 400 mg/kg were revealed. Increase in the frequency of diffuse atrophy of the seminiferous tubules, hyperplasia of Leydig cells in the testis, decreased spermatozoa and residual

Table 6. Organ weights for F1 male parents exposed to BBP.

			BBP (mg/kg/day)			
			0	100	200	400
No. of animals examined			14	19	16	13
Terminal weight (g)			645.4 ± 93.3	644.0 ± 70.1	633.6 ± 55.7	617.9 ± 61.1
Liver		(g) [a]	23.10 ± 3.49	24.19 ± 3.77	24.29 ± 2.28	25.94 ± 3.21
		(g/100 g) [b]	3.56 ± 0.24	3.74 ± 0.24	3.84 ± 0.23*	4.19 ± 0.23**
Kidney	right	(g)	2.05 ± 0.28	2.06 ± 0.29	2.04 ± 0.34	2.05 ± 0.25
		(g/100 g)	0.32 ± 0.03	0.32 ± 0.03	0.32 ± 0.04	0.33 ± 0.02
	left	(g)	1.99 ± 0.26	1.98 ± 0.27	1.99 ± 0.30	1.99 ± 0.26
		(g/100 g)	0.31 ± 0.03	0.31 ± 0.03	0.31 ± 0.03	0.32 ± 0.02
Testis	right	(g)	1.93 ± 0.17	1.92 ± 0.12	1.92 ± 0.14	1.84 ± 0.58
		(g/100 g)	0.30 ± 0.04	0.30 ± 0.04	0.31 ± 0.03	0.30 ± 0.09
	left	(g)	1.91 ± 0.20	1.92 ± 0.12	1.86 ± 0.18	1.81 ± 0.27
		(g/100 g)	0.30 ± 0.04	0.30 ± 0.04	0.30 ± 0.03	0.29 ± 0.04
Epididymis	right	(g)	0.72 ± 0.06	0.71 ± 0.09	0.67 ± 0.04	0.60 ± 0.17*
		(g/100 g)	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.01	0.10 ± 0.03
	left	(g)	0.73 ± 0.07	0.68 ± 0.08	0.64 ± 0.07*	0.61 ± 0.10**
		(g/100 g)	0.11 ± 0.01	0.11 ± 0.02	0.10 ± 0.01	0.10 ± 0.02
Ventral prostate		(g)	0.69 ± 0.22	0.70 ± 0.17	0.63 ± 0.10	0.60 ± 0.16
		(g/100 g)	0.11 ± 0.03	0.11 ± 0.03	0.10 ± 0.02	0.10 ± 0.02
Seminal vesicle		(g)	1.86 ± 0.19	1.77 ± 0.19	1.69 ± 0.14	1.62 ± 0.29*
		(g/100 g)	0.29 ± 0.05	0.28 ± 0.05	0.27 ± 0.03	0.26 ± 0.04
Brain		(g)	2.17 ± 0.08	2.14 ± 0.08	2.12 ± 0.09	2.09 ± 0.09
		(g/100 g)	0.34 ± 0.04	0.34 ± 0.04	0.34 ± 0.03	0.34 ± 0.04
Spleen		(g)	0.90 ± 0.24	0.85 ± 0.14	0.84 ± 0.13	0.83 ± 0.10
		(g/100 g)	0.14 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.13 ± 0.02
Pituitary		(mg)	15.2 ± 2.4	14.8 ± 1.7	14.2 ± 2.0	14.4 ± 2.0
		(mg/100 g)	2.4 ± 0.4	2.3 ± 0.3	2.3 ± 0.2	2.3 ± 0.4
Thyroid		(mg)	25.9 ± 7.2	26.0 ± 5.6	26.6 ± 5.9	31.3 ± 6.9
		(mg/100 g)	4.1 ± 1.2	4.1 ± 0.9	4.2 ± 0.8	5.1 ± 0.9*
Adrenal gland	right	(mg)	33.5 ± 5.8	30.2 ± 3.6	30.3 ± 3.2	32.7 ± 4.3
		(mg/100 g)	5.2 ± 0.7	4.7 ± 0.7	4.8 ± 0.4	5.3 ± 0.7
	left	(mg)	35.1 ± 7.1	32.2 ± 3.7	32.7 ± 3.7	33.7 ± 4.8
		(mg/100 g)	5.4 ± 0.8	5.0 ± 0.8	5.2 ± 0.6	5.5 ± 0.7

Values are mean ± SD.
[a] Absolute weight.
[b] Relative weight.
* Significantly different from the controls at $p<0.05$.
** Significantly different from the controls at $p<0.01$.

germ cells in the epididymal lumina, and bilateral or unilateral partial aplasia, or unilateral aplasia of the epididymis have all been reported to be effects of BBP (Agrawal *et al*., 1985; Piersma *et al*., 1995; Nagao *et al*., 2000, Tyl *et al*., 2004), and were reproduced in this study. Such changes in F1 parents were found at a lower dose than in F0 parents, and *in utero* exposure was assumed to be a possible cause. The morphological changes of the testis and epididymis in F1 males receiving of 400 mg/kg were similar to those in rats administered 2,3,7,8-tetrachlorobenzodioxin (TCDD) during the middle gestational phase (Wilker *et al*., 1996; Suzuki, 2000). With phthalate esters, the mechanisms of toxicity for testis have not been clarified, but they might be similar the TCDD case. As for the small size or softening of the testes and hyperplasia of Leydig cells, it is known that similar morphological anomalies can be observed in rats with hereditary autoimmune disease (Musto *et al*., 1978). The trend for decrease in absolute splenic weight observed in F1 males of 400 mg/kg group may indicate that BBP effects some immunological change. It should be noted in this context that no effect was found in Sertoli's cells of the testis in either generation in the present study.

Significant lowering body weights were found in F1 male offspring receiving 100 mg/kg or more. In F2 offspring, significantly similar changes were found in males of 100 mg/kg group and in females of 100 and 400 mg/kg groups. However, the changes in F2 offspring were transient and lacked dose-dependence, and

Table 7. Organ weights for F1 female parents exposed to BBP.

			BBP (mg/kg/day)			
			0	100	200	400
No. of animals examined			15	19	15	12
Terminal weight (g)			316.0 ± 21.1	329.0 ± 20.2	336.0 ± 30.1	320.4 ± 32.2
Liver		(g) [a]	12.79 ± 1.29	13.15 ± 1.24	13.74 ± 1.65	14.01 ± 1.45
		(g/100 g) [b]	4.04 ± 0.23	3.99 ± 0.28	4.09 ± 0.34	4.38 ± 0.24**
Kidney	right	(g)	1.10 ± 0.15	1.20 ± 0.11	1.23 ± 0.20	1.16 ± 0.16
		(g/100 g)	0.35 ± 0.03	0.36 ± 0.03	0.37 ± 0.04	0.36 ± 0.03
	left	(g)	1.09 ± 0.15	1.16 ± 0.11	1.17 ± 0.22	1.15 ± 0.17
		(g/100 g)	0.34 ± 0.03	0.35 ± 0.03	0.35 ± 0.05	0.36 ± 0.04
Ovary	right	(g)	56.0 ± 8.4	62.6 ± 11.1	57.4 ± 10.3	51.5 ± 12.1
		(g/100 g)	17.7 ± 2.2	19.0 ± 3.1	17.1 ± 0.3	16.1 ± 3.3
	left	(g)	56.2 ± 7.8	62.0 ± 9.1	57.6 ± 8.7	53.2 ± 9.5
		(g/100 g)	17.8 ± 2.3	18.8 ± 2.7	17.2 ± 2.6	16.6 ± 2.2
Uterus		(g)	0.74 ± 0.11	0.75 ± 0.09	0.80 ± 0.09	0.83 ± 0.15
		(g/100 g)	0.24 ± 0.03	0.23 ± 0.03	0.24 ± 0.03	0.26 ± 0.04
Brain		(g)	1.95 ± 0.09	1.96 ± 0.08	1.96 ± 0.06	2.01 ± 0.09
		(g/100 g)	0.62 ± 0.50	0.60 ± 0.50	0.59 ± 0.06	0.63 ± 0.06
Spleen		(g)	0.55 ± 0.06	0.56 ± 0.05	0.56 ± 0.06	0.54 ± 0.08
		(g/100 g)	0.18 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.02
Pituitary		(mg)	17.2 ± 3.3	17.7 ± 2.1	17.5 ± 2.4	15.9 ± 1.7
		(mg/100 g)	5.4 ± 0.9	5.4 ± 0.7	5.2 ± 0.7	5.0 ± 0.4
Thyroid		(mg)	22.6 ± 3.5	23.7 ± 7.7	22.1 ± 5.4	22.4 ± 4.3
		(mg/100 g)	7.2 ± 1.2	7.2 ± 2.3	6.6 ± 1.4	7.0 ± 1.4
Adrenal gland	right	(mg)	33.2 ± 5.7	34.6 ± 4.9	32.4 ± 3.4	34.8 ± 4.1
		(mg/100 g)	10.5 ± 1.6	10.6 ± 1.7	9.7 ± 1.5	10.9 ± 1.1
	left	(mg)	34.9 ± 5.7	37.1 ± 4.5	35.1 ± 4.0	36.9 ± 3.5
		(mg/100 g)	11.0 ± 1.5	11.3 ± 1.7	10.5 ± 1.7	11.6 ± 1.2

Values are mean ± SD.
[a] Absolute weight.
[b] Relative weight.
** Significantly different from the controls at $p<0.01$.

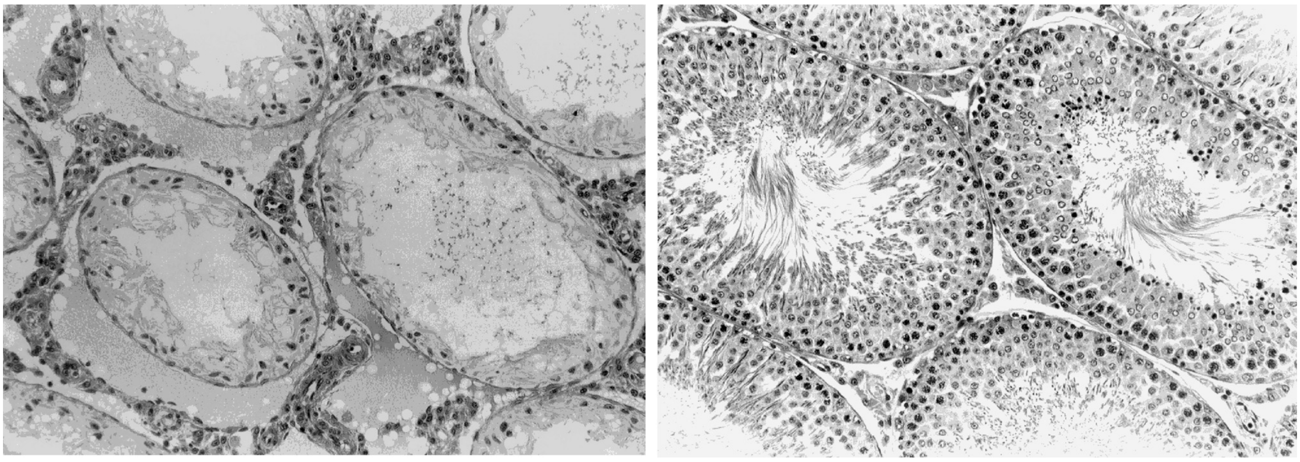

Photo 1. Left: Diffuse atrophy of seminiferous tubules and Leydig cell hyperplasia at the dose of 400mg/kg/day. H&E, ×20. Right: Control testis. H&E, ×20.

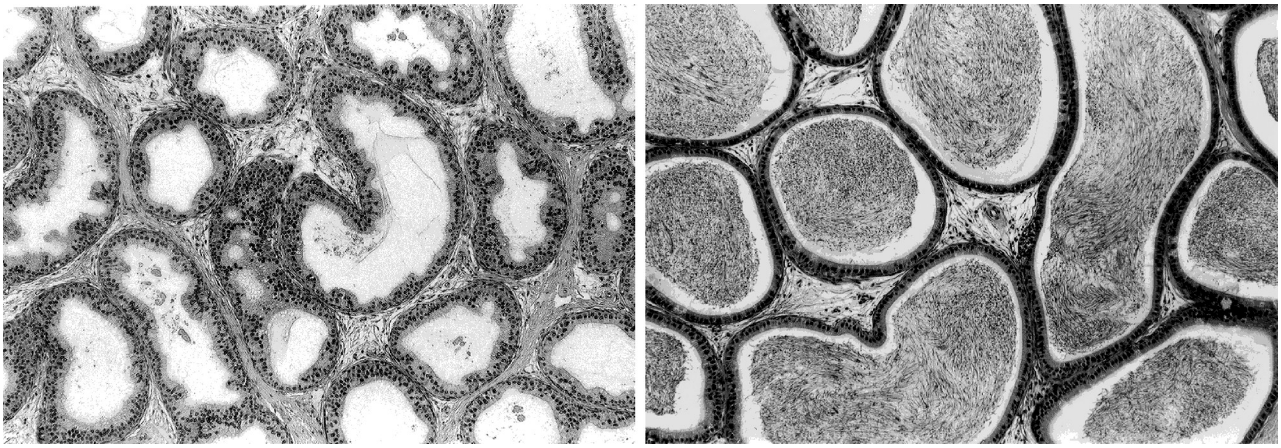

Photo 2. Left: Decreased spermatozoa and residues of germ cells in the epididymal lumina at the dose of 400mg/kg/day. H&E, ×10. Right: Control epididymis. H&E, ×10.

Table 8. Autopsy findings for F1 male rats (adult) exposed to BBP.

	BBP (mg/kg/day)			
	0	100	200	400
No. of animals examined	24	24	24	24
Testis				
Small	0	0	0	6*
Softening	0	1	2	4
Epididymis				
Aplasia	0	0	0	1
Hypoplasia	0	0	0	4
Small	0	0	0	3

Values are frequencies.
* Significantly different from the controls at $p<0.05$.

are not, thus, considered due to BBP.

No abnormality was found in the sex ratio of the pups delivered, viability, and external surfaces in F1 and F2 offspring. While increase in AGD (mm/cubic root of body weight) was observed in the F1 female pups in the groups given 100 mg/kg or more, there was

Table 9. Histopathological findings for F1 male rats (adult) exposed to BBP.

	BBP (mg/kg/day)			
	0	100	200	400
No. of animals examined	24	24	24	24
Testis				
Diffuse atrophy of seminiferous tubules	1	1	3	9*
Leydig cell hyperplasia	0	1	0	5*
Epididymis				
Decreased spermatozoa in lumina	0	1	2	3
Residus of germ cells in lumina	0	1	3	1
Partial aplasia (bilateral)	0	0	0	1
Partial aplasia (unilateral)	0	0	0	3
Aplasia (unilateral)	0	0	0	2

Values are frequencies.
* Significantly different from the controls at p<0.05.

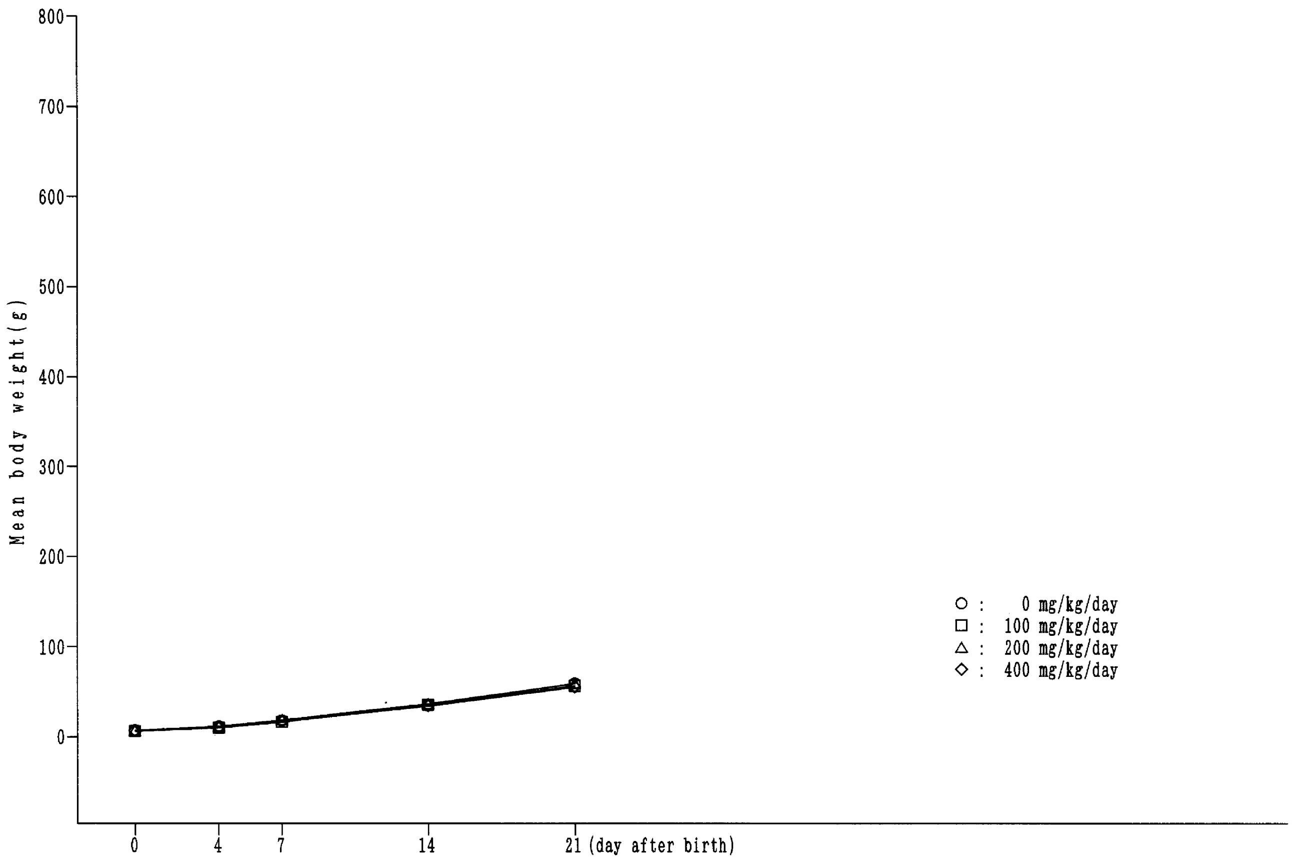

Fig. 3. Body weight of F2 male rats exposed to BBP.

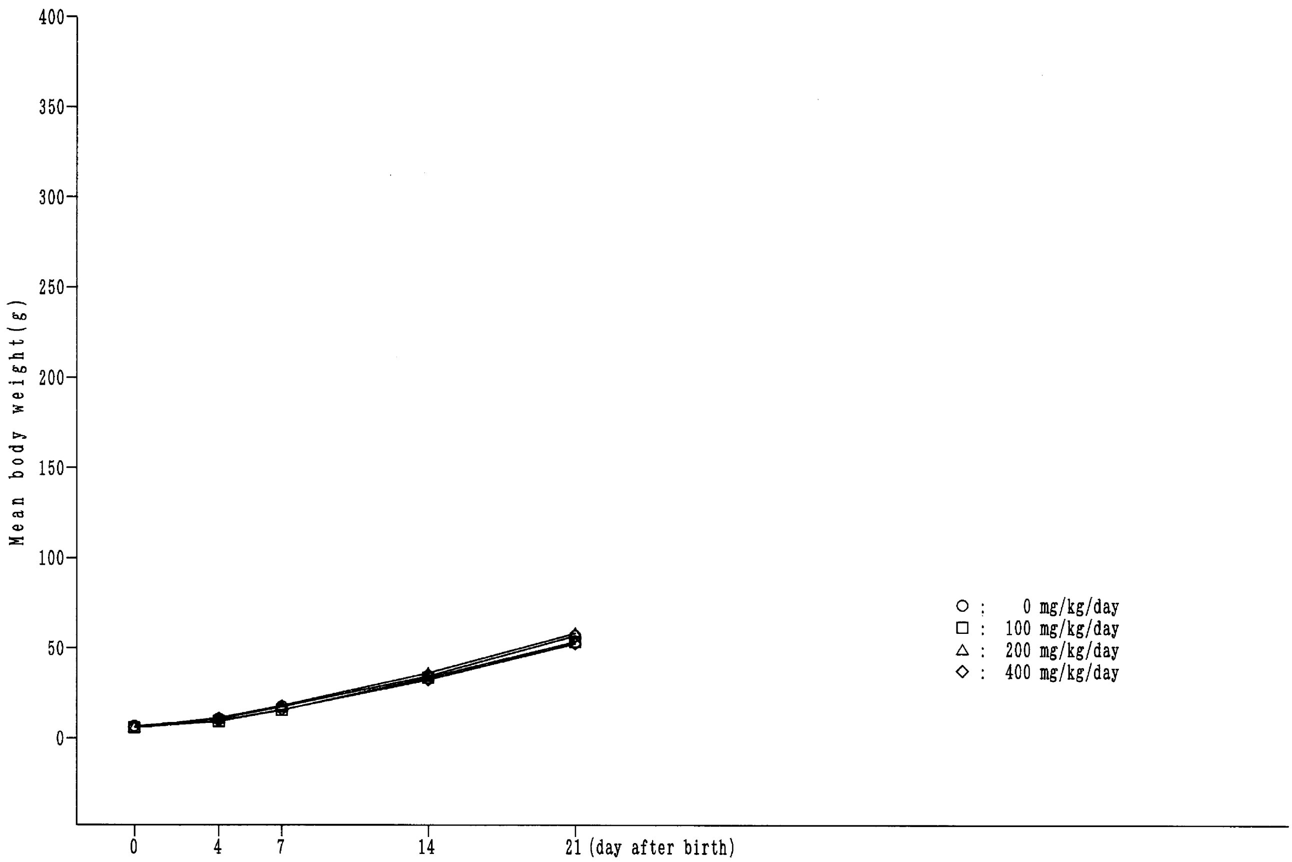

Fig. 4. Body weight of F2 female rats exposed to BBP.

Table 10. Physical development of F1 offspring exposed to BBP.

Sex		BBP (mg/kg/day) 0	100	200	400
Male	Anogenital distance (mm)	4.44 ± 0.50	4.47 ± 0.42	4.34 ± 0.41	4.31 ± 0.50
	(mm/$^3\sqrt{}$ BW)	2.02 ± 0.20	2.02 ± 0.16	2.00 ± 0.18	1.98 ± 0.19
	Pinna unfolding	112/112 (100)	106/106 (100)	137/137 (100)	124/124 (100)
	Incisor eruption	68/72 (94.4)	57/68 (83.8)	74/78 (94.9)	71/75 (94.7)
	Eye opening	72/72 (100)	65/68 (95.6)	74/78 (94.9)	74/75 (98.7)
Female	Anogenital distance (mm)	2.12 ± 0.20	2.34 ± 0.19*	2.29 ± 0.29	2.24 ± 0.29
	(mm/$^3\sqrt{}$ BW)	0.98 ± 0.08	1.08 ± 0.09**	1.07 ± 0.13*	1.06 ± 0.10*
	Pinna unfolding	139/139 (100)	116/116 (100)	120/120 (100)	129/129 (100)
	Incisor eruption	79/83 (95.2)	75/76 (98.7)	71/73 (97.3)	80/82 (97.6)
	Eye opening	83/83 (100)	76/76 (100)	69/73 (94.5)	80/81 (98.8)

Values are mean ± SD.
Values in parentheses are percentages.
* Significantly different from the controls at p<0.05.
** Significantly different from the controls at P<0.01.

Table 11. Physical development of F2 offspring exposed to BBP.

Sex		BBP (mg/kg/day) 0	100	200	400
Male	Anogenital distance (mm)	4.75 ± 0.43	4.20 ± 0.33**	4.39 ± 0.56	4.08 ± 0.55**
	(mm/$^3\sqrt{}$ BW)	2.12 ± 0.16	1.96 ± 0.11*	1.94 ± 0.16**	1.87 ± 0.21**
	Pinna unfolding	95/95 (100)	128/128 (100)	70/70 (100)	78/78 (100)
	Incisor eruption	54/58 (93.1)	63/72 (87.5)	39/43 (90.7)	46/49 (93.9)
	Eye opening	56/58 (96.6)	72/72 (100)	42/43 (97.7)	49/49 (100)
Female	Anogenital distance (mm)	2.30 ± 0.29	2.09 ± 0.21	2.16 ± 0.28	2.24 ± 0.23
	(mm/$^3\sqrt{}$ BW)	1.05 ± 0.11	1.00 ± 0.09	0.96 ± 0.09	1.06 ± 0.10
	Pinna unfolding	88/88 (100)	129/129 (100)	70/70 (100)	69/69 (100)
	Incisor eruption	52/57 (91.2)	68/79 (86.1)	57/60 (95.0)	43/47 (91.5)
	Eye opening	57/57 (100)	76/79 (96.2)	60/60 (100)	47/47 (100)

Values are mean ± SD.
Values in parentheses are percentages.
* Significantly different from the controls at p<0.05.
** Significantly different from the controls at p<0.01.

Table 12. Organ weights for F1 male offspring exposed to BBP.

		BBP (mg/kg/day) 0	100	200	400
No. of animals examined		19	18	19	20
Terminal weight (g)		58.4 ± 3.4	59.9 ± 5.2	58.4 ± 4.4	57.1 ± 4.2
Brain	(mg) [a]	1498.8 ± 42.4	1507.3 ± 45.1	1513.9 ± 67.2	1506.2 ± 55.1
	(mg/100 g) [b]	2571.3 ± 130.0	2531.5 ± 195.0	2601.4 ± 189.4	2650.4 ± 200.4
Spleen	(mg)	294.0 ± 67.2	296.1 ± 67.7	303.8 ± 53.9	247.6 ± 39.8*
	(mg/100 g)	499.7 ± 92.9	491.5 ± 91.0	518.4 ± 76.2	433.1 ± 56.2*
Thymus	(mg)	247.0 ± 32.2	263.0 ± 43.1	259.7 ± 39.5	262.8 ± 37.7
	(mg/100 g)	422.3 ± 44.9	439.6 ± 65.5	443.9 ± 54.3	459.4 ± 50.1

Values are mean ± SD.
[a] Absolute weight.
[b] Relative weight.
* Significantly different from the controls at p<0.05.

Table 13. Organ weights for F2 male offspring exposed to BBP.

		BBP (mg/kg/day) 0	100	200	400
No. of animals examined		15	19	14	12
Terminal weight (g)		59.1 ± 5.5	56.8 ± 6.7	59.1 ± 9.4	55.0 ± 6.2
Brain	(mg) [a]	1493.9 ± 52.9	1454.7 ± 60.0	1476.3 ± 63.4	1493.8 ± 50.7
	(mg/100 g) [b]	2539.8 ± 176.0	2587.5 ± 240.3	2542.1 ± 324.8	2739.8 ± 244.5
Spleen	(mg)	305.4 ± 65.5	268.1 ± 70.7	281.5 ± 63.1	229.4 ± 54.5*
	(mg/100 g)	514.2 ± 89.5	466.5 ± 79.1	472.8 ± 47.6	413.5 ± 58.8**
Thymus	(mg)	244.0 ± 31.2	242.2 ± 45.0	250.6 ± 53.1	225.3 ± 49.9
	(mg/100 g)	412.9 ± 44.3	427.5 ± 69.0	422.4 ± 53.0	406.4 ± 51.3

Values are mean ± SD.
[a] Absolute weight.
[b] Relative weight.
* Significantly different from the controls at p<0.05.
** Significantly different from the controls at p<0.01.

no clear dose-dependence and the same change was not observed in the F2 pups, and the values were actually within the normal range for this strain. However, since it has been reported that increase in female AGD is an effect of BBP (Nagao *et al*., 2000), it cannot be denied that the changes was effects of this substance. Similarly the decrease in AGD (mm/cubic root of body weight) in males is in line with the literatures (Ashby *et al*., 1997; Nagao *et al*., 2000; Ema and Miyawaki, 2002; Tyl *et al*., 2004).

Lowering splenic weights in the F1 and F2 male offspring observed at the dose of 400 mg/kg were considered to be slight, since no changes for pathological findings or organ weights were found in the F1 parents.

No abnormalities in clinical signs, physical development, response test or gross appearance were considered to be caused by BBP in the F1 and F2 offspring of either sex in any dose group. Retention of nipples and areolae in male offspring with administration of 750 mg/kg (Gray *et al*., 2000) or 11,250 ppm in the diet (approximately 750 mg/kg/day) (Tyl *et al*., 2004), and thus presence or absence of the effects might be attributed to the dose difference or different administration route.

In the present study, extra parameters such as estrous cyclicity, vaginal opening and preputial separation of the penis, as well as sperm indices, weight data, histopathological features of reproductive and endocrine organs, AGD, adopted in the proposal for updating guideline 416 of OECD were added, along with measurement of hormone levels. Although not all changes of BBP reported previously were reproduced, we could here detect the effects reduced fertility, delayed preputial separation of the penis, epididymal malformation, histopathological changes in the testis and epididymis, and decreased AGD and epididymal weights in the males. Thus the utility of this protocol for detecting non-steroid chemicals with endocrine activity was confirmed.

In conclusion, when BBP was administered over two generations to rats in the present study, the no observed effect level (NOEL) and the no observed adverse effect level (NOAEL) for the parental animals were both less than 100 mg/kg/day. With regard to their reproductive capacity, the dose of 400 mg/kg caused reduction of the fertility index and delayed preputial separation of the penis in the males. However, no marked effects were evident at doses less than 200 mg/kg/day. With regard to the NOEL and NOAEL for development and growth in the offspring, it is concluded that these values are also less than 100 mg/kg/day.

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