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Inhalation of 1-Bromopropane Causes Excitation in the Central Nervous System of Male F344 Rats

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Abstract

The present study investigates the effects of 1-bromopropane (1BP) on animal behavior to determine the extent of toxicity to the central nervous system (CNS). We measured the spontaneous locomotor activity (SLA) of rats before and after 3 weeks of exposure to 1BP for 8 h per day. In control and 10 ppm groups, the SLA values were similar to pre-exposure levels on post-exposure Day 1 and thereafter. However, the SLA values in the 50 and 200 ppm groups were higher than pre-exposure levels. Open-field behavior was evaluated after exposure and freezing time decreased with exposure to increasing concentrations of 1BP. Ambulation and rearing scores in the exposed groups were higher than control values, particularly in the 50 and 200 ppm groups. The frequency of defecation and urination decreased almost dose-dependently. Exposure to 50–1000 ppm of 1BP did not affect passive avoidance behavior examined using a step-through type apparatus. The amount of time swimming in the water maze test was not affected in the controls, or groups exposed to 50 and 200 ppm 1BP, but that in the 1000 ppm group was increased compared with control. Exposure at 50–1000 ppm dose-dependently decreased the traction performance of rats, indicating decreased muscle strength. We found that 10–200 ppm of 1BP exposure did not affect motor coordination determined by rota-rod performance. The increased SLA values and open-field activity support the notion that 1BP has excitatory effects on the CNS of F344 male rats. In addition, 1BP reduced the grip or muscle strength of the rats. Memory function was not disordered and the motor coordination of all four limbs remained normal.

Keywords: 1-Bromopropane; Behavior; Central nervous system; Toxicity; Rats

INTRODUCTION

The semiconductor industry uses 2-bromopropane (2BP) as an ozone-depleting substance replacement (ODSR). In 1995, Korean workers exposed to severe 2BP intoxication in an electronics factory developed reproductive and hematopoietic disorders (Kim et al., 1996). Male workers developed oligozoospermia or azoospermia, females developed amenorrhea, and pancytopenia was evident in both. However, the frequency of hematopoietic disorders was lower than that of reproductive disorders. Therefore, the effects on reproductive functions were considered to be the most serious toxic effects of 2BP in humans. The reproductive toxicity of 2BP has been confirmed by animal experiments. Sperm counts are reduced in male rats injected with 2BP or exposed to 2BP gas (Ichihara et al., 1997). Exposure to 2BP disturbs the estrous cycle and elicits a decrease in the number of ovulated ova in female mice and rats (Sekiguchi and Honma, 1998; Sekiguchi et al., 2001, 2002). The reproductive and hematopoietic toxicity of 2BP has led to a decrease in its use and replacement with 1-bromopropane (1BP). However, the results of animal experiments indicate that 1BP is toxic to the peripheral nerves (Ichihara et al., 2000; Yu et al., 1998). The grip strength of all four limbs of male Wistar rats decreased

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following exposure to 800 ppm of 1BP for 8 h per day for 8 weeks. Maximum motor nerve conduction velocity decreased and distal latency increased in the tail nerve at 800 ppm. Histopathological changes were evident in the peripheral nerve and muscle fibers. On the other hand, the effects of 1BP on the central nervous system (CNS) have been poorly characterized.

The present study investigates the effects of 1BP on animal behavior to determine the extent of CNS toxicity using a combination of relatively simple standard procedures. Motor activity, open-field behavior, memory tests such as passive avoidance and maze are standard tests used to evaluate CNS effects. The traction test detects disorders in peripheral nerves or the muscular system and the rota-rod test detects changes in motor coordination.

MATERIALS AND METHODS

Animals

Male 8-week-old F344 rats obtained from Charles River Japan Inc. were acclimatized in stainless steel wire net cages in groups of five per cage for at least 14 days with a light/dark cycle of 12 h:12 h (lights on at 8:00 h) The temperature and humidity in the breeding and exposure rooms were maintained at 23 ± 1 °C and 55 ± 5%, respectively. Food (CE-2, Japan Clea Inc.) and water were accessible ad libitum. Behavioral effects were measured in different groups of rats.

Experimental design and chemicals

After acclimatization, rats were grouped such that mean body weight did not differ among them. The rats inhaled vaporized 1BP (GR grade, Tokyo Chemical Industry Co. Ltd., Japan) in stainless steel chambers (Sibata Inc., Tokyo) as described (Sekiguchi et al., 2002; Tsuga and Honma, 2000). The exposure concentration of 1BP (10, 50, 200, and 1000 ppm) (50, 251, 1006, and 5028 mg/m^3, respectively) was monitored using a gas chromatograph (Shimadzu GC-7A, Japan) every 15 min and adjusted with flowmeters to a constant target value of ±5% throughout the study. Control rats were exposed to clean air. The rats were exposed to 1BP for 8 h every day between 0:00 and 8:00 h for 3 weeks so that the results could be compared with those of 2BP toxicity. We selected doses of up to 1000 ppm in the present study because in a previous investigation of reproductive toxicity, we exposed rats to a maximum of 1000 ppm of 1BP and 2BP for 3 weeks (Sekiguchi et al., 2002).

Body weight and temperature

As fundamental physiological indices, we weighed the animals and measured body temperature using a thermometer (model MGA-III, Type 219, Nihon Koden, Japan) equipped with a rectal thermo probe. These indices were obtained from the same rats between 10:00 and 12:00 h each day.

Locomotor activity

To examine spontaneous locomotor activity (SLA), the animals were individually housed in plastic home cages (38 cm width × 22 cm depth × 15 cm height) and SLA was measured before and after exposure to 1BP. Motility was determined as the amount of thermal radiation emitted by each caged rat, using an infrared sensor mounted above the cage (Supermex; Muromachi Kikai Co. Ltd., Japan) (Masuo et al., 1997). We used eight infrared sensors to simultaneously measure the SLA of eight rats. As two rats each were used for each dose, exposure was limited to four maximal doses including the control for the SLA study. Therefore, 10, 50, and 200 ppm were the exposure concentrations because body weight decreased during exposure to 1000 ppm for 3 weeks.

Open-field test

Behavior was observed and scored in a square (90 cm × 90 cm) arena surrounded by 50-cm high walls and separated into 25 equal squares (Honma and Kitagawa, 1977). At the beginning of the test, each rat was placed in the center of the arena and the freezing time (latency before leaving the central square) was measured. The number of square borders crossed was counted and recorded as the ambulation score. The frequency of rearing, preening (grooming and face washing), defecation, and urination episodes was counted and recorded. Behavior was observed for 3 min and each score was manually recorded. Rats quickly become habituated to the open-field arena and behavior scores significantly decreased after repeating the test even after several days. Therefore, each rat was tested only once in the open-field arena.

Passive avoidance test

Passive avoidance was examined using a step-through type apparatus (Muromachi Kikai Co. Ltd., Japan) (Liu et al., 1999). At the beginning of the experiment, a rat was placed in a light room and

electroshocked with 0.2 mA for 5 s from the floor grid (mode 1) the moment the rat entered a dark room (pre-exposure conditioning). Three or 4 days later, the rat was put in the light room and the latency before entering the dark room was measured and recorded. When the rat did not enter the dark room within 10 min, the measurement was ceased and 10 min was recorded as latency. The mode 1 conditioning procedure was repeated four times before exposure. Through these procedures, the latency of almost all of the rats exceeded 10 min (600 s). In the afternoon of each exposure day, rats were subjected to the passive avoidance test between 13:00 and 14:00 h. During and after 3 weeks of exposure, this test was repeated and the electroshock was not given even if the rat entered the dark room within 10 min (mode 2). In the other experiment, rats were exposed to 1BP for 3 weeks without passive avoidance conditioning. On the day of final exposure (post-exposure Day 0), between 13:00 and 14:00 h, the rats were conditioned in the passive avoidance apparatus with electroshocks (post-exposure conditioning). On post-exposure Day 1 or later, we measured the latency required to enter the dark room using mode 2.

Water maze test

The Morris water maze test was performed in a 1.5 m diameter circular water pool (Muromachi Kikai Co. Ltd., Japan) that was filled to a depth of 30 cm with tap water at 25 °C (Morris, 1984). A transparent circular escape platform, 12 cm diameter, was placed 1 cm below the surface of the water. Before exposure to 1BP, rats were trained by swimming with six daily trials (three times each in the morning and afternoon) of 2 min each to reach the platform. The platform was placed in one quadrant of the pool. The rat was placed in one of the other three quadrants and positioned to face the wall. If the rat could not reach the hidden platform within 2 min, it was removed to avoid overload and sinking. Three quadrants where the platform was not placed were used as the initial quadrant placement for rats each morning and afternoon. When all rats could reach the platform within 30 s, they were exposed to 1BP (pre-exposure learning). Latency before reaching the platform was measured during and after exposure, between 10:00 and 15:00 h. In another experiment, rats were exposed to 1BP for 3 weeks without pre-exposure training in the water maze. On the day of final exposure (post-exposure Day 0) or later, rats were placed in the water pool for training (post-exposure learning). Latency before reaching the platform was measured and recorded.

Traction test

A plastic bar, 3 mm in diameter, was set horizontally 50 cm over the desk surface (Kuribara et al., 1977; Morimoto and Kito, 1994). A rat was forced to hang in the air from the bar with the fore-limbs. After confirming that the grasp was sufficient, the rat was left hanging. The time until the rat fell from the bar was recorded. The trial was repeated three times, between 13:00 and 15:00 h, and the longest period of hanging was considered as traction time.

Rota-rod test

Rats were placed on a rod, 9 cm diameter rotating at 5 rpm, and were trained to stay on the bar for at least 3 min (Dunham and Miya, 1957; Kuribara et al., 1977; Morimoto and Kito, 1994). When all rats could remain on the rotating rod for almost 3 min, they were exposed to 1BP. Measurement of the amount of time the animal remained on the rod was ceased at 3 min. This test was applied between 13:00 and 15:00 h.

Statistics

Dunnett's multiple t-test in a statistical program (SPSS Japan Inc.) compared controls and each of the exposed groups. Rats were repeatedly subjected to the passive avoidance, water maze, traction and rota-rod tests at intervals of a few days. A repeated measures ANOVA using StatView® for Windows (Hulinks Inc., Japan) analyzed the dose and repeated effects of exposure in these tests to detect differences among groups. Differences between groups at $P < 0.05$ were considered significant.

RESULTS

Body weight and temperature

The rats were weighed every Monday, Wednesday, and Friday of the experimental period. Mean body weights of the control, 10, 50, 200, and 1000 ppm groups on the day before exposure were almost identical ($N = 5$). Fig. 1 shows the time course of changes in weight up to 21 days post-exposure. The weight of control rats and of those exposed to 10 or 50 ppm of 1BP similarly increased over time. The amount of weight gained by rats exposed to 200 ppm of 1BP was greater than that of the control group, becoming statistically significant from exposure Day 11

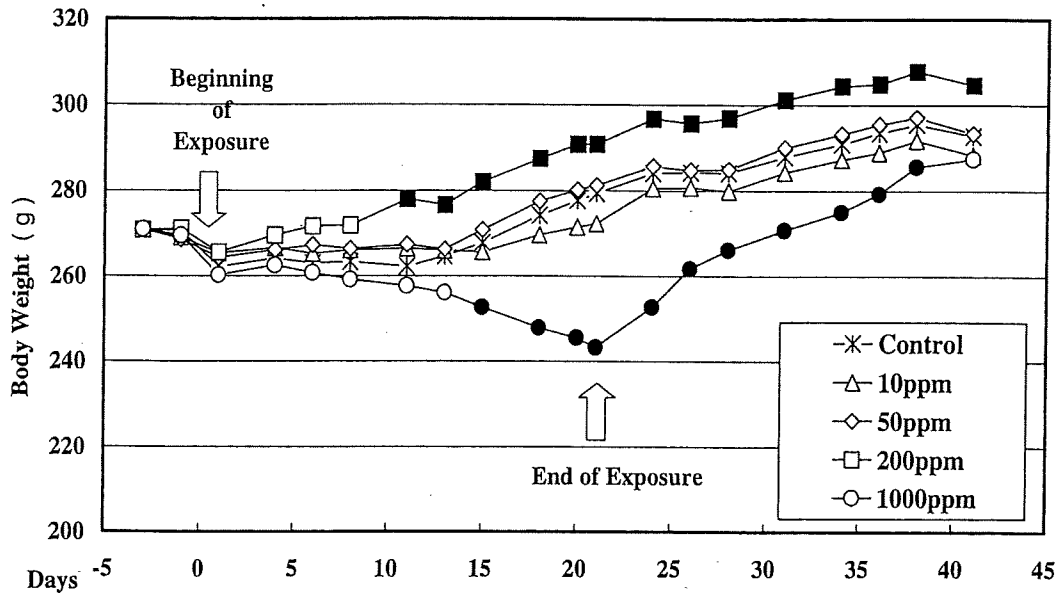

Fig. 1. Time-course changes in body weight of male F344 rats exposed to 1-bromopropane. Mean body weights were determined in each group exposed to 0 (control), 10, 50, 200, and 1000 ppm. Closed marks indicate significant differences from control values at each exposure day at $P < 0.05$ detected with Dunnett's test.

(Dunnett's test). These differences were maintained even at 3 weeks after stopping 1BP inhalation. The weight of the 1000 ppm group decreased gradually over time and became statistically different from that of the control from exposure Day 15. This weight loss was recovered after inhalation was stopped and the weight of the 1000 ppm and control groups did not significantly differ at 3 weeks after the end of exposure.

Fig. 2 shows the time course of changes in body temperatures up to 28 post-exposure days. Exposure to 1BP reduced body temperature In particular, 1000 ppm significantly lowered the body temperature at 1–7 exposure days according to Dunnett's test ($N = 5$). These effects almost totally disappeared after the cessation of exposure.

Locomotor activity

Before exposure to 1BP, rats were individually placed in plastic home cages under infrared light detectors. The SLA values were recorded every

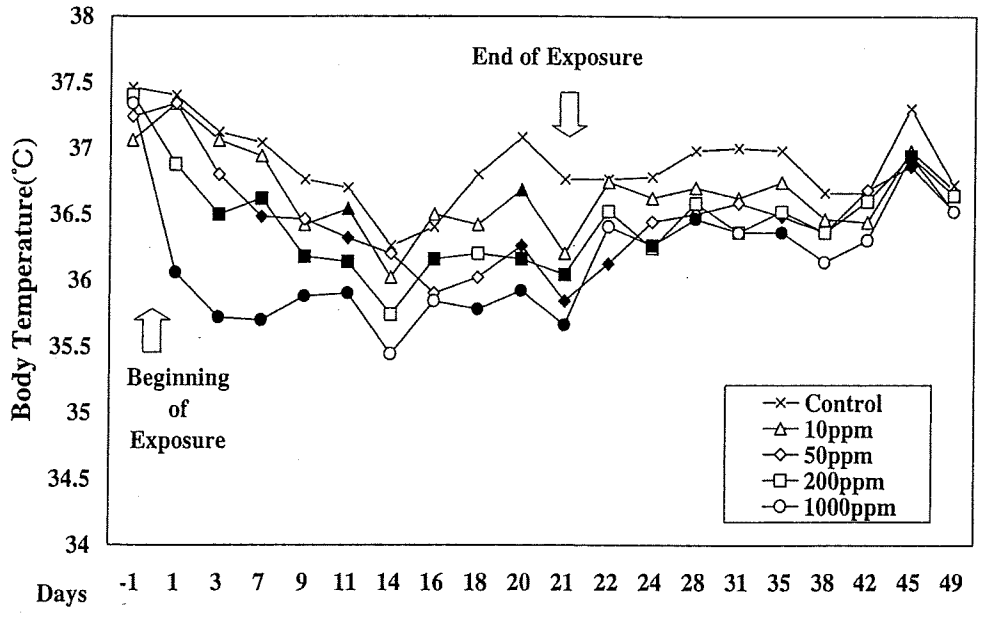

Fig. 2. Time-course changes in body temperature of rats exposed to 1-bromopropane. Closed marks in each exposure group indicate significant difference from control values ($P < 0.05$).

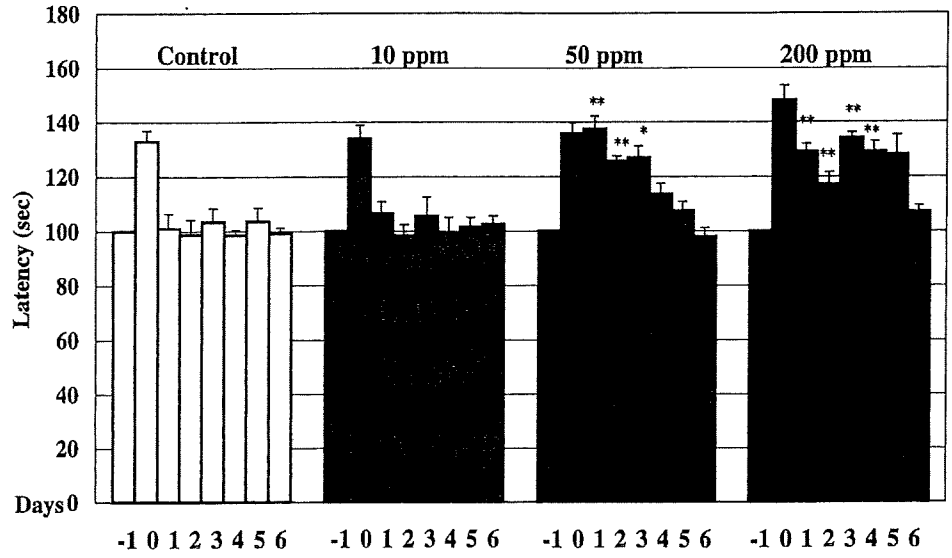

Fig. 3. Effects of 1-bromopropane exposure for 3 weeks on spontaneous locomotor activity of rats during post-exposure dark period. Pre-exposure level was set as 100% for each rat. Mean activity and S.E.M. were calculated from four rats per exposure group and statistical significance of differences from control each day was detected with Dunnett's test. Vertical bars indicate S.E.M. values. (*) $P < 0.05$; (**) $P < 0.01$.

30 min for at least 2 weeks to determine acclimatization. When the SLA values had stabilized, the rats were transferred to exposure chambers. At 8:00 h on the last day of exposure, the animals were returned to the plastic cages and SLA was measured (Day 0) every 30 min to determine mean SLA values during dark (20:00–8:00 h) and light (8:00–20:00 h) periods. The mean SLA values of individual rats on the last day before starting exposure were separately set at 100% for each of the dark and light periods. Fig. 3 presents the mean SLA counts of the dark period obtained from four rats in each group following 3 weeks of exposure. On Day 0, the SLA counts of the dark period were higher than pre-exposure levels in all four groups. The SLA counts of the control and 10 ppm groups returned to pre-exposure levels from Day 1. However, the SLA counts of the 50 and 200 ppm groups were higher than pre-exposure levels on Day 1 and persisted for 3–4 days. Differences between control and 50 or 200 ppm groups were statistically significant during Days 3–4 post-exposure (Dunnett's test). To identify the effects of a single 8-h exposure to 1BP on motility, four groups of rats were exposed to 0, 50, 200, and 1000 ppm of 1BP. Fig. 4 shows the effects of a single 8-h exposure to 1BP on SLA counts. In control and 50 ppm groups, SLA counts on the Day 0 were slightly below those at

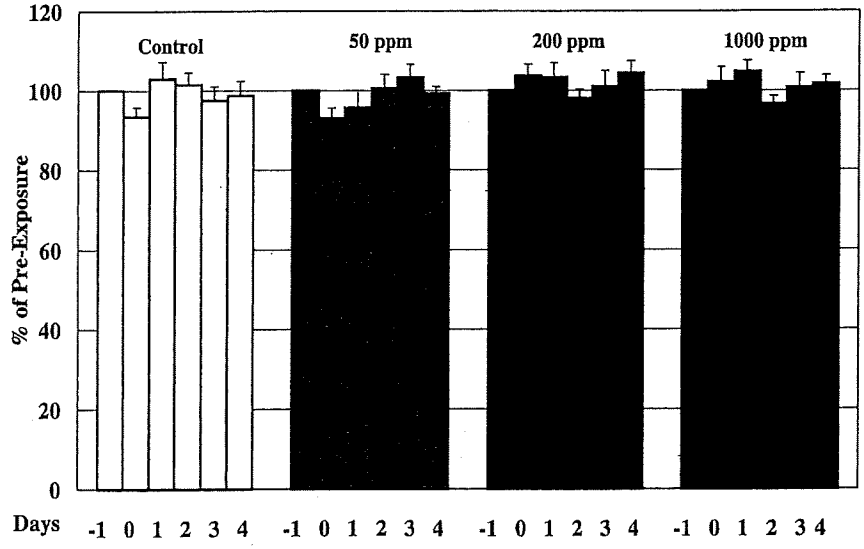

Fig. 4. Effects of a single 8-h dose of 1-bromopropane on spontaneous locomotor activity of rats during post-exposure dark period (see legend to Fig. 3).

pre-exposure, but those from Day 1 were almost identical to pre-exposure counts. In 200 and 1000 ppm groups, SLA counts after exposure were almost identical to pre-exposure levels for 4 days post-exposure. The differences between SLA values of control and exposed groups after a single 8-h exposure were not statistically significant.

Open-field test

Rats were divided into five groups and exposed to 0, 10, 50, 200, or 1000 ppm of 1BP for 3 weeks. On the day after the last exposure, the rats underwent the open-field test between 10:00 and 12:00 h. Mean scores of each behavior were obtained from five rats in each group (Fig. 5). Freezing time dose-dependently decreased at 50–1000 ppm, although the findings were not statistically different according to Dunnett's test. Ambulation increased with increasing 1BP concentration, but the score at 1000 ppm was lower than that at 200 ppm. A significant increase compared to control was obtained at 200 ppm. The relationship between exposure concentration and rearing score was similar to that between the 1BP concentration and ambulation score. Exposure to 1BP did not affect preening behavior. Scores of defecation and urination were included. The defecation + urination score was reduced by exposure and the difference was statistically significant from control at 1000 ppm.

Other groups of rats received single exposure to 1BP at 50–1000 ppm for 8 h and then underwent the open-field test on the following day. Mean behavioral scores for each of four groups were determined from five rats (Fig. 6). The ambulation and rearing score tended to increase according to increasing exposure concentrations, but the differences were not statistically significant. Preening, defecation, and urination scores were not affected by 1BP.

Passive avoidance test

After undergoing pre-exposure conditioning, almost all rat stayed in the light room for at least 10 min. Therefore, mean latency of six rats in each group required to enter the dark room was almost 10 min. Five groups of rats were exposed to 0, 10, 50, 200, or 1000 ppm of 1BP for 3 weeks. The test was repeated during the exposure period and mean latency at each test is shown in Fig. 7. Apart from the initial conditioning process, no electroshocks were administered to the rats when they entered the dark room. Therefore, latency became shorter with repeated tests with extinction of the memory of shocks. The repeat effects were statistically significant according to ANOVA ($F(6,24) = 63.508$, $P < 0.0001$). Latency between control and exposed groups did not differ throughout the study at all exposure concentrations (ANOVA and Dunnett's test).

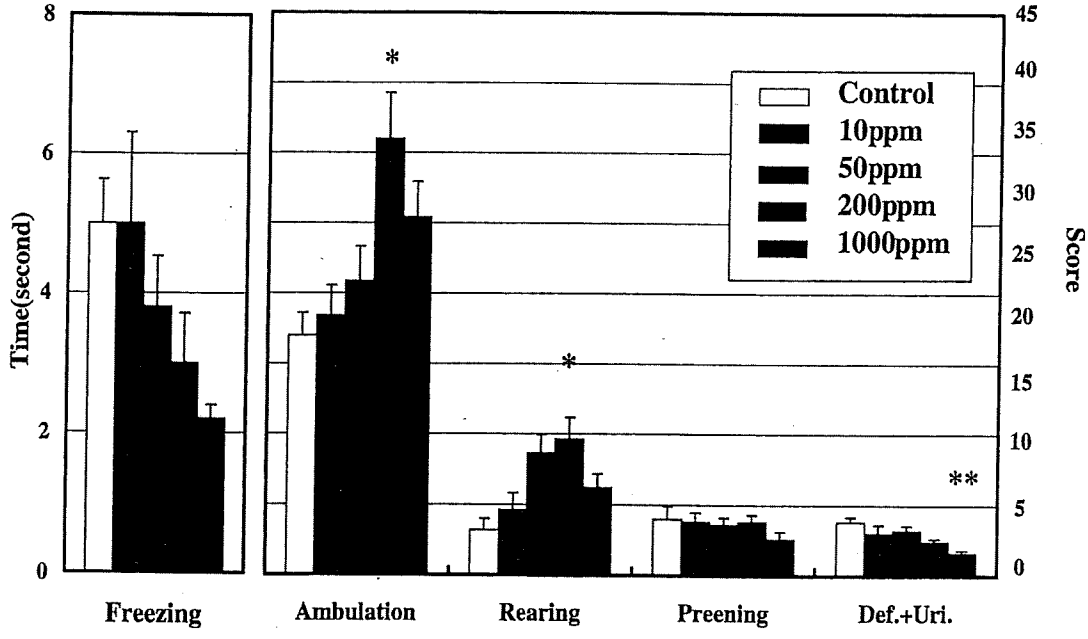

Fig. 5. Effects of 1-bromopropane exposure for 3 weeks on open-field activity of rats. Mean and S.E.M. values of freezing time or behavioral scores were calculated for each exposure group and statistical significance of differences from control was detected with Dunnett's test. Vertical bars indicate S.E.M. values. (*) $P < 0.05$; (**) $P < 0.01$.

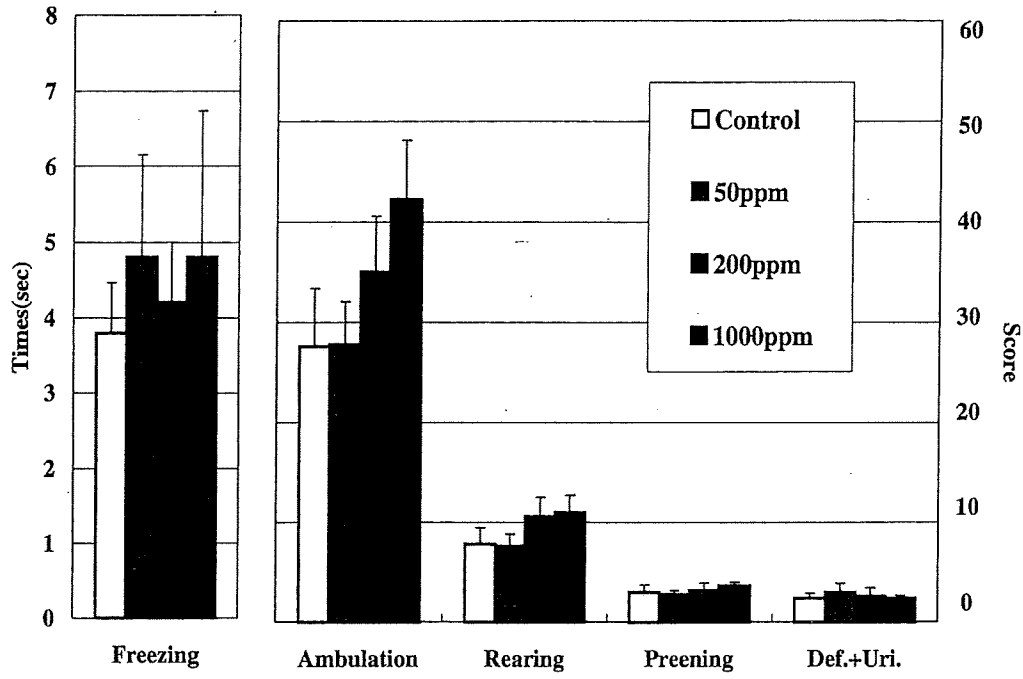

Fig. 6. Effects of a single 8-h dose of 1-bromopropane on open-field activity of rats (see legend to Fig. 5).

Fig. 8 shows the results of passive avoidance after post-exposure conditioning in rats exposed to 0, 50, 200, and 1000 ppm 1BP for 3 weeks. On post-exposure Days 1 and 3, the mean latency of three exposed groups to enter the dark room was shorter than that of the control ($N = 5$). However, the differences were not statistically significant (Dunnett's test). ANOVA revealed a significant repeat effect during post-exposure Days 1 and 7 ($F(3, 16) = 47.11$, $P < 0.0001$). Three weeks of exposure to 1BP did not obviously affect the acquisition of avoidance.

Water maze test

After pre-exposure learning for 2 weeks, mean latency (swimming time) required to reach the platform

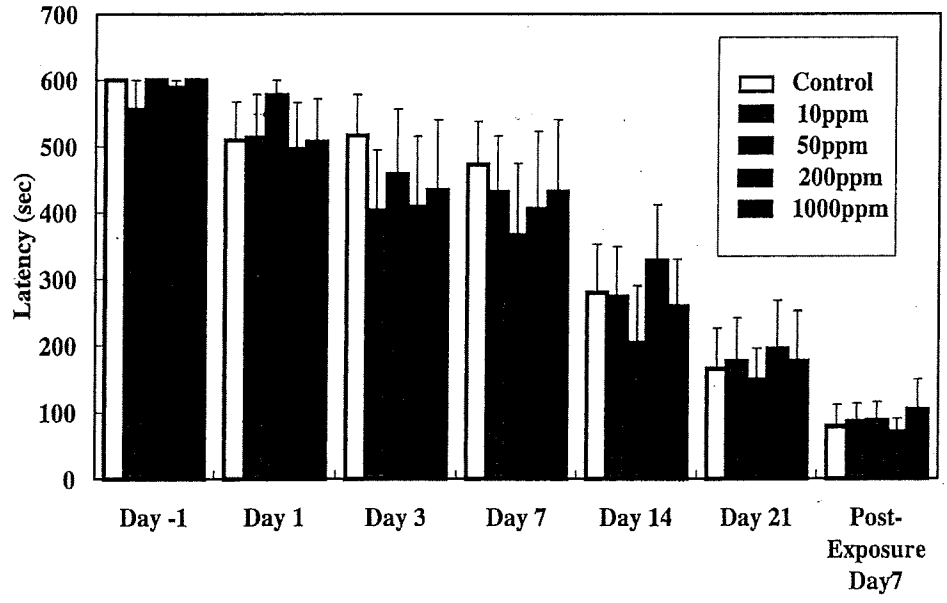

Fig. 7. Effects of 1-bromopropane exposure for 3 weeks on rat passive avoidance. Rats were conditioned to avoid electroshock before exposure and avoidance was tested during and after exposure (pre-exposure conditioning). Mean and S.E.M. values of latency to enter the dark room were calculated for each exposure group.

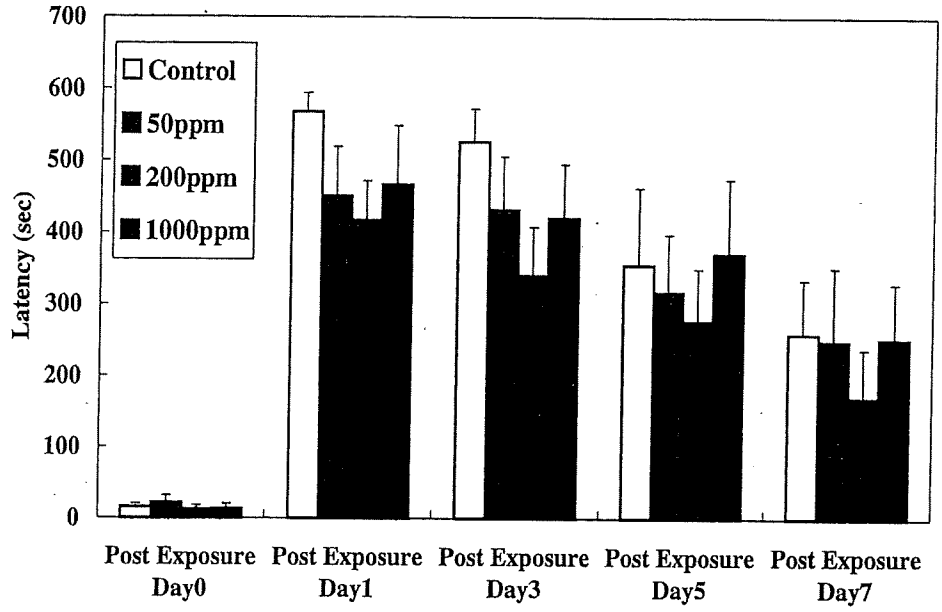

Fig. 8. Effects of 1-bromopropane exposure for 3 weeks on rat passive avoidance. Rats were conditioned after exposure (post-exposure conditioning). Mean and S.E.M. values of latency were calculated for each group.

was between 5 and 10 s for each rat. These rats were then exposed to 0, 10, 50, 200, or 1000 ppm of 1BP for 3 weeks. Mean latency for each rat was determined from six trials per day and that of each group was determined from five rats per group. Fig. 9 shows, that 10 and 50 ppm of exposure did not affect latency, whereas 200 and 1000 ppm prolonged latency in groups of five rats. Dose effects were not significant according to ANOVA ($F(4,20) = 1.240$, $P = 0.326$). However, the repeat effects were statistically significant ($F(6,20) = 3.465$, $P < 0.003$). At 21 days of exposure, the difference in latency between the 1000 ppm and control groups became statistically significant (Dunnett's test).

The effects of 1BP at 50, 200, and 1000 ppm on the post-exposure learning schedule are shown in Fig. 10. On post-exposure Day 0, mean latency in the three exposed groups was longer than that in control, but escape learning in the water maze was not significantly affected by 3 weeks of 1BP exposure as revealed by

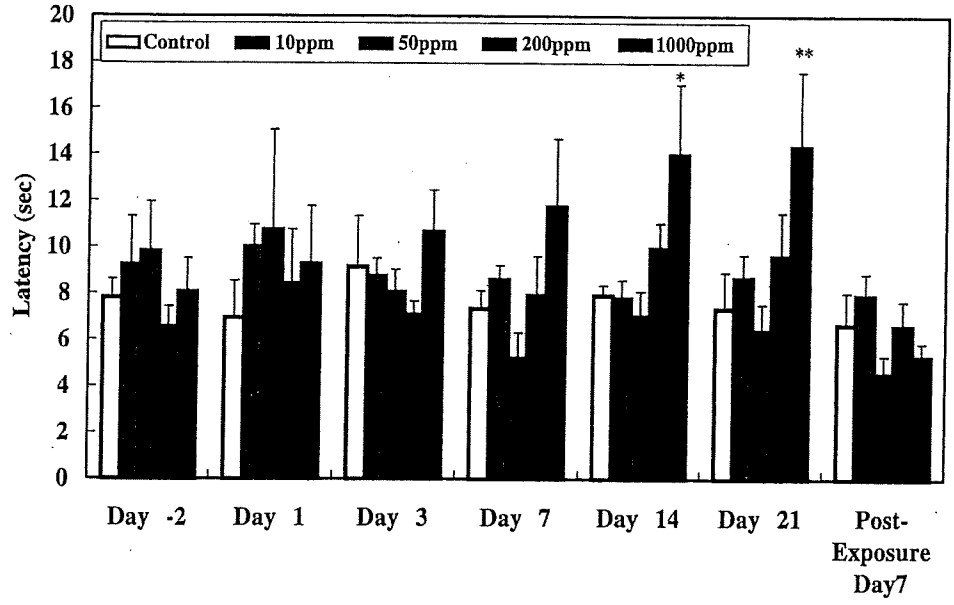

Fig. 9. Effects of exposing rats to 1-bromopropane for 3 weeks on water maze performance. Rats were trained to reach an escape platform before exposure then subjected to water maze testing during and after exposure (pre-exposure learning). Mean and S.E.M. values of latency required to reach the escape platform were calculated for each exposure group and statistical significance of differences from control was detected with Dunnett's test. (*) $P < 0.05$; (**) $P < 0.01$.

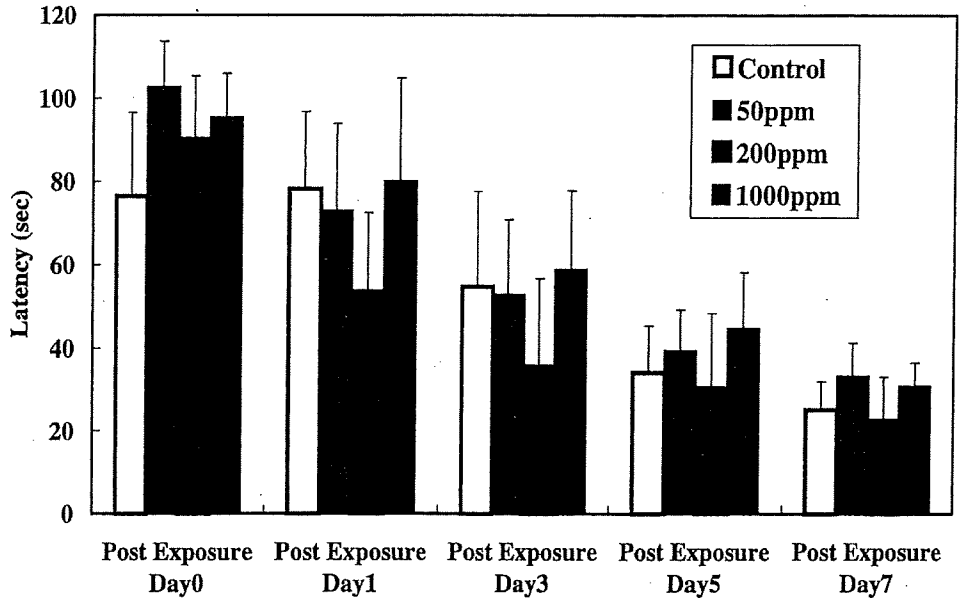

Fig. 10. Effects of exposing rats to 1-bromopropane for 3 weeks on water maze performance. Rats were trained to reach an escape platform after exposure (post-exposure learning) (see legend to Fig. 9).

Dunnett's test ($N = 5$). Latency decreased with repeated trials in all of four groups and repeat effects were significant ($F(4, 16) = 22.040$, $P < 0.0001$).

Traction test

The traction test was performed before and after the beginning of exposure. Five groups of five rats each inhaled 1BP at 0, 10, 50, 200, or 1000 ppm for 3 weeks. After 7 days of exposure, the traction time in higher exposure concentration groups was shorter than in groups exposed to lower concentrations (Fig. 11). ANOVA revealed that the dose effects were significant ($F(4, 20) = 12.747$, $P < 0.0001$). After 2 weeks of exposure, the traction time of 1000 ppm group was significantly shorter than control (Dunnett's test). On the last day of the 3-week exposure, differences from control were statistically significant in the 200 and 1000 ppm groups. Even at 7 days after the cessation of exposure, the traction times of the 200 and 1000 ppm groups were significantly shorter than those of the control group. Repeat effects during six repeated

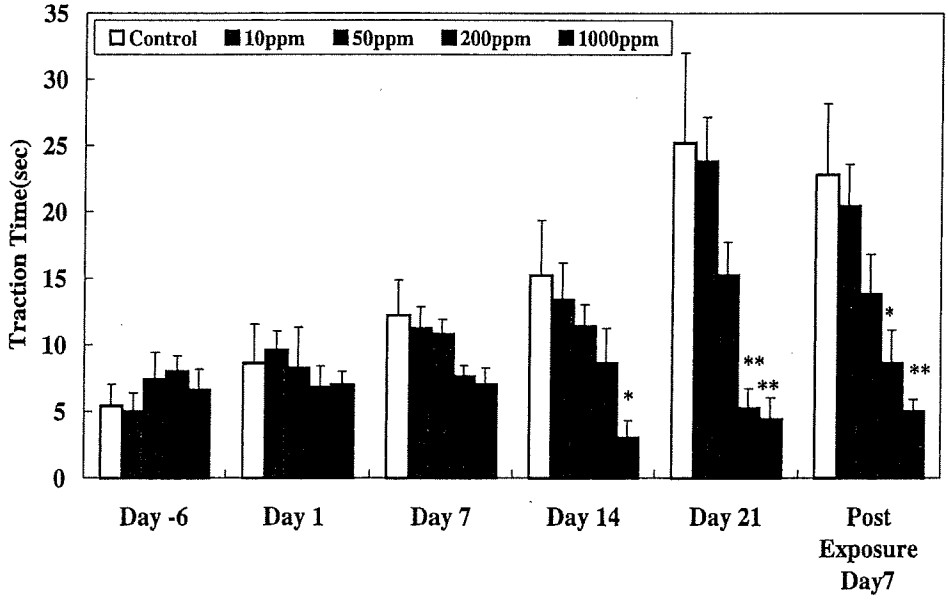

Fig. 11. Effects of exposing rats to 1-bromopropane for 3 weeks on traction time. Mean and S.E.M. values of traction time were calculated for each exposure group and statistical significance of differences from control was detected with Dunnett's test. (*) $P < 0.05$; (**) $P < 0.01$.

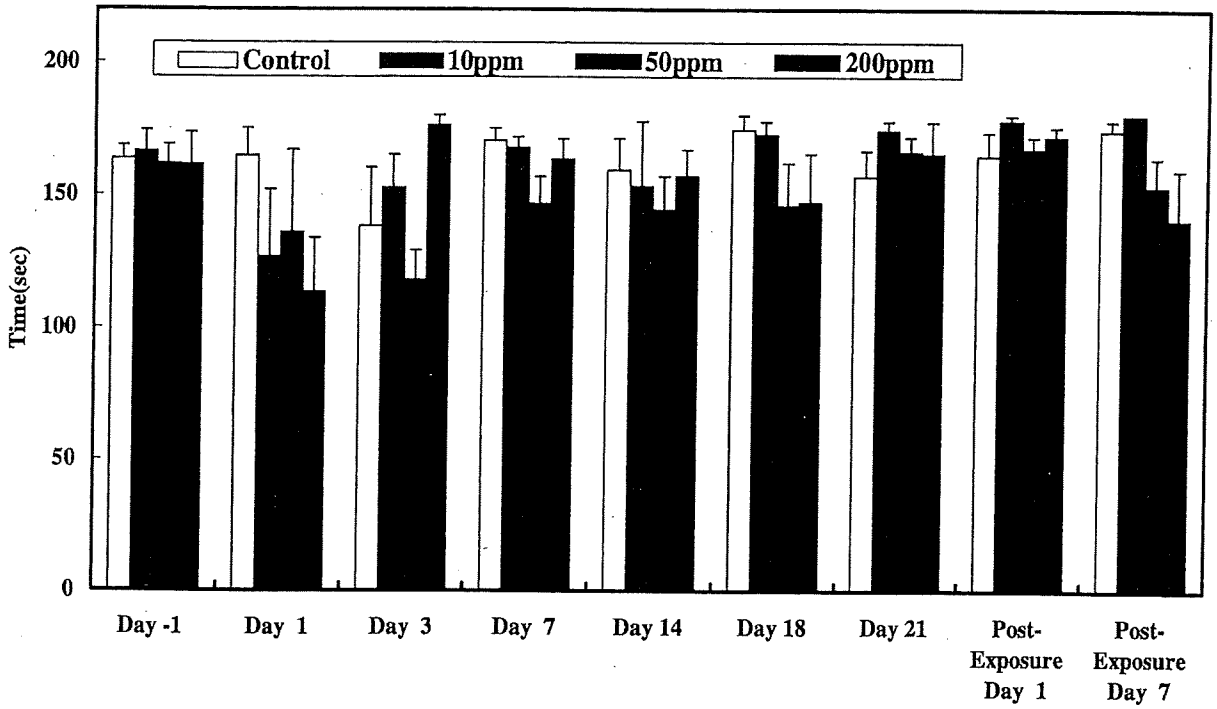

Fig. 12. Effects of exposing rats to 1-bromopropane for 3 weeks on rota-rod performance. Mean and S.E.M. values of time remaining on a rotating rod were calculated for each group.

measurements were significant ($F(5,20) = 7.847$, $P < 0.0001$).

Rota-rod test

We measured the amount of time that groups of five rats remained on the rod. After the first exposure to 1BP, the 10, 50, and 200 ppm groups remained for a somewhat shorter period on the rod, but these effects disappeared at 7 exposure days (Fig. 12). No statistical significance in dose effects was revealed by ANOVA ($F(3,14) = 2.083$, $P = 0.149$) and the amount of time remaining on the rod did not differ between the control and all of the exposed groups even on the last day of the 3-week exposure (Dunnett's test). Repeating the test improved rota-rod performance and repeat effects during all measurements were statistically significant according to ANOVA ($F(8,14) = 2.787$, $P = 0.008$).

DISCUSSION

The results of other studies of 1BP toxicity are summarized in Table 1. Some present experimental evidence that 1BP is toxic to the peripheral nerves, whereas studies of humans and other animals suggest that 1BP is toxic to the CNS.

The present study found that exposure to 10 and 50 ppm of 1-BP for 8 h per day for 3 weeks induced no significant differences in body weights from the control throughout the study. However, the 200 ppm group gained significantly more weight during the exposure period than the control group. We found that the 200 ppm group consumed more food than the control group (data not shown). We previously exposed female rats to 50–1000 ppm 1BP for 8 h per day for 3 weeks to determine the effect on female reproductive functions. In that study, the exposed groups also gained more weight than controls although the difference was not significant (Sekiguchi et al., 2002). The present study found that exposure to 1000 ppm 1BP reduced the body weight of male rats compared with control (Fig. 1). Food intake of rats in the 1000 ppm group was suppressed during the exposure (data not shown). The weight gain of female rats exposed to 1000 ppm of 1BP was somewhat less than that of the 200 ppm group, but greater than that of control rats (Sekiguchi et al., 2002). Although 1BP increased the appetite of rats, it might be suppressive at high doses in both male and female rats. Such effects of 1BP seem to be more potent in male, than in female rats. The weight of female rats exposed to 2BP at 50–1000 ppm for 3 weeks (Sekiguchi et al., 2002) increased more than that of control group. Although the mechanism of the increase in appetite and body weight is unclear, the mechanisms of the effects of 1BP and 2BP might be similar. To date, whether industrial chemicals can increase food intake has remained unknown. The

Table 1
Studies of effects of 1BP on humans and experimental animals[a]

Subject	Exposure to 1BP	Effect	Reference
Rats	Inhalation, 4 weeks 1500 ppm	Loss of body weight, ataxic gait Degeneration of cerebellar Purkinje cells	Ohnishi et al. (1999)
Humans	Exposure, 2 months	Weakness of lower extremities and right hand, numbness, dysphagia; symmetrical demyelinating polyneuropathy; patchy areas of increased T2 signal in periventricular white matter	Sclar (1999)
Rats	Inhalation, 12 weeks 200–800 ppm	Decreased epididymal sperm count and motility Failure of spermiation	Ichihara et al. (2000)
Rats	Inhalation, 12 weeks 200–800 ppm	Decreased grip strength in fore- and hind-limbs Deterioration of MCV and DL of the tail nerve	Ichihara et al. (2000)
Rats	Inhalation, 3 weeks 50–1000 ppm	Changes in estrous cycle	Sekiguchi et al. (2002)
Rats	Inhalation, 7 days 200–800 ppm	Decrease in brain gamma-enolase activity	Wang et al. (2002)
Rats	Inhalation, 13 weeks 200–1250 ppm	No pathological changes to gray and white matter	Sohn et al. (2002)

[a] Subjects, exposure profiles, and effects identified in the referenced studies.

mechanism of an increase in food intake caused by 1BP remains to be clarified.

The body temperature of rats was dose-dependently decreased by exposure to 1BP, particularly during the first 7 days of the exposure period, and this became remarkable at 1000 ppm. Hypothermia frequently develops in animals exposed to organic solvents and might be closely associated with the anesthetic action of volatile chemicals. A decline of consciousness at 1000 ppm or more of 1BP was notable, although normothermia gradually recovered with repeated exposure.

The increase in SLA among rats exposed to 50 and 200 ppm 1BP lasted for 3–4 days after exposure stopped, indicating that 1BP molecules remaining in the body after the exposure did not produce the increase. The concentration of 1BP in the rat brain at 4 h after ceasing exposure was about 5% of that during exposure and below the limits of detection at 8 h after exposure (Suda and Honma, unpublished data). The effects of toluene on the muscarinic acetylcholine receptors of the rat brain last after toluene has disappeared from the brain tissue (Tsuga and Honma, 2000; Tsuga et al., 1999). Exposure to 1BP might have caused functional or biochemical changes lasting for 3–4 days in the neuronal system of the brain. The increase in SLA due to 1BP exposure was reversible because SLA was not increased at 6 days after exposure. The SLA was not affected by a single 8-h exposure to 1BP. Repeated exposure to 1BP is required to increase SLA in rats. Because of circadian rhythms, the SLA value of rats in the dark period is three to five times greater than that in the light period. An injection of a serotonin depleter such as *p*-chlorophenylalanine (Fuller, 1980; Honma, 1978) causes these rhythms in the SLA values of rats to disappear. However, these rhythms were maintained in rats exposed to 1BP and unchanged as revealed by circadian rhythm analysis (data not shown) even after 3 weeks of exposure. These results suggest that 1BP can excite the CNS without altering circadian rhythms. Because SLA during the dark period was much greater than that during the light period, the effects of chemicals on this value in the dark period can be more easily detected.

In the open-field test, 1BP exposure for 3 weeks non-significantly reduced freezing time and also reduced defecation + urination scores compared with controls. Ambulation and rearing scores increased in groups exposed to 1BP. Almost all of these behavioral changes were dose related. Excitatory drugs such as methamphetamine reduces freezing time and increases ambulation and rearing scores (Honma and Kitagawa, 1977). Anxiolytic drugs such as benzodiazepine tranquilizers reduce emotional defecation and urination of rats in the open-field situation (Honma and Kitagawa, 1977). Our results suggest that 1BP stimulates exploration and reduces anxiety in rats facing novel circumstance. While weight gain and appetite increased at 200 ppm and decreased at 1000 ppm, decreases in freezing time, defecation and urination frequency were dose related. These results suggest that the changes in these behaviors were not affected by the decreases in weight gain and appetite at 1000 ppm. Following a single 8-h exposure to 1BP, ambulation and rearing scores tended to increase in the 200 and 1000 ppm groups, but the differences were not

statistically significant. Stimulation of exploratory behavior and reduction of emotional anxiety produced in rats by exposure to 1BP in the open-field situation were enhanced by repeated exposure. The mechanisms of these effects of 1BP are unclear and remain to be elucidated.

In the passive avoidance and water maze tests, a 3-week exposure to 1BP produced no effects except in water maze performance at 1000 ppm that might have resulted from the impaired muscular systems observed in the traction test. The maintenance of acquired memory in male rats is probably not affected by 1BP. In post-exposure conditioning or learning schedules, 1BP exposure did not affect passive avoidance and water maze performance. Exposing rats to 1BP for 3 weeks did not affect the acquisition process of avoidance or learning behavior.

The present study showed that 1BP exposure remarkably reduced the traction time following repeated exposure. Others have reported that 1BP has peripheral nerve toxicity. Motor nerve conduction velocity is decreased and myelin sheaths become enlarged following 5 or 7 weeks of exposure to 1000 ppm (Yu et al., 2001). The effects of 1BP on traction time in our experiments must be due to peripheral nerve toxicity. We observed statistically significant effects of 1BP in male F344 rats 2 weeks after the beginning of exposure at 1000 ppm or after 3 weeks of exposure at 200 ppm. Despite being toxic to the peripheral nerves, 1BP did not affect motor coordination as demonstrated by rota-rod performance, suggesting that it does not affect the motor control system in the CNS.

Ohnishi et al. (1999) exposed male Wistar rats to 1BP for 6 h per day, 5 days per week, for 4 weeks at 1500 ppm. During the latter half of the experiment, the gait of all rats exposed to 1BP became ataxic. These exposure conditions were similar to those used in the present study. The decreased traction time found in our study after 2 weeks of exposure to 1000 ppm may be closely related to the ataxic gait described by Ohnishi et al. (1999). According to Ichihara et al. (2000), hind-limb grip strength of male Wistar rats exposed to 1BP for 8 h per day for 12 weeks was lowered in 200–800 ppm groups compared with control after 4 weeks of exposure. Fore-limb grip strength was weakened in 400 and 800 ppm groups following 8 weeks of exposure. Overall, the 1BP exposure conditions that produced effects on grip strength were similar to those that affected traction time in our experiment. The action mechanism of 1BP might be common to ataxic gait, weakened grip strength, and decreased traction time.

According to Sohn et al. (2002), exposing male SD rats to 1250 ppm 1BP for 6 h per day, 5 days per week for 13 weeks produced no pathological changes in either gray or white matter of the brain. We believe that functional investigations like those performed in the present study can evaluate the neurotoxicity of chemicals using relatively simple experimental procedures.

The increases in SLA, stimulation of exploratory behavior, and reduction of emotional anxiety identified in the open-field test after a 3-week exposure to 1BP support the notion that 1BP has excitatory effects on the CNS of male F344 rats. If the peripheral nerve toxicity of 1BP affected behavior, SLA and exploratory behaviors in the open-field situation would have been suppressed. To elucidate the mechanism of these effects, a neurochemical approach is now in progress. The passive avoidance and rota-rod results were not affected by exposure to 1BP. Water maze performance was disturbed only at 1000 ppm of 1BP and these effects disappeared at 7 days after exposure was stopped. These results indicate that the memory and learning function of rats is not disordered and that the coordination of all four limbs is not disturbed by 1BP. Further studies with additional measures of investigation are required to more precisely define the neurotoxicity of the ODSR compound, 1BP.

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Short Communication

Imbalance of Testosterone Level in Male Offspring of Rats Perinatally Exposed to Bisphenol A

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Abstract: The purpose of this study was to investigate whether exposure to bisphenol A (BPA) through the placenta and milk has any effect on the reproductive system in male offspring. Pregnant rats were treated with BPA at 0, 4, 40 and 400 mg/kg body weight, from gestation day 6 through lactation day 20 by gavage. Plasma testosterone concentrations in offspring at 9 weeks old were significantly high in BPA groups as compared with those of the control. At the age of 36 weeks the hormone concentrations showed an increase in a dose-dependent manner, although without statistical significance. Testosterone content in testes showed a similar tendency to that in plasma, though statistically insignificant. Little alteration in testes weight was seen in BPA-exposed offspring. There was no remarkable change in plasma concentrations of luteinizing hormone and follicle-stimulating hormone at 9 weeks old. The pathway of E_2 (17β-estradiol) formation from testosterone seemed not to be affected by BPA. The results indicate that exposure to BPA during the perinatal period has a significant effect on testosterone homeostasis in male offspring of rats.

Key words: Bisphenol A, Testosterone, Offspring, Perinatal exposure, Male rat

Bisphenol A (BPA) is a widely used industrial material, and more than 150 tons are manufactured annually in Japan (Ministry of International Trade and Industry, 1999). It is mainly used as a fungicide, antioxidant, and stabilizer in rubber and plastic products. BPA monomer has been found to be released and migrate from cans coated with epoxy or polyvinylchloride resins and from polycarbonate tableware and baby bottles[1,2]. It is also a component of dental sealants, and has been found in the saliva of dental patients treated with such sealants[3]. The toxicity of this compound, including its effect as an endocrine disruptor, has been of great concern because of its occupational exposure and intake in daily life.

It has been reported that BPA could bind to estrogen receptors both *in vitro* and *in vivo*, though much less potent than E_2 (17β-estradiol), and therefore mimic the effects of female hormone[4,5]. The compound has been detected in umbilical cord blood and mother's milk[6,7], and one could easily imagine the possibility of its effects on fetuses and newborns. Sex hormones, particularly testosterone, are critical for the differentiation and development of the brain, reproductive organs and other systems in the perinatal period, and the disorder of this hormone during this period may induce irreversible changes in reproductive organs or function at mature ages.

Fetuses and newborn are believed to be much more sensitive to chemical exposure than adults. There have been some studies on laboratory animals, but the results are controversial and the effects of BPA on offspring are still unclear. Prenatal exposure to BPA was reported to result in an increase in prostate weight, or less sperm production at mature ages[8,9]. In other reports, no BPA dependent effects were found in male offspring with regard to the weight of sex organs or other indices[10,11]. By extending the period of BPA administration, and with a wide range of doses in the present study, we found a significant effect of BPA exposure on testosterone homeostasis in the male offspring of rats at pubertal age.

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Pregnant rats (Crj: CD (SD) IGS strain, 9 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan) at gestation day 3. They were housed individually with a light/dark cycle of 12/12 h (light on at 8.00a.m.) Room temperature and humidity were maintained at 23 ± 1°C and 55 ± 5%, respectively. Feed (CE-2, Clea Japan, Inc.) and water were accessible *ad libitum*. The rats were divided into four groups at random and given BPA with a purity of >99.8% (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 0 (control), 4, 40, and 400 mg/kg body weight (BW) completely dissolved in corn oil, from gestation day 6 through lactation day 20 by gavage. The number of offspring was standardized to ten (male:female=5:5, where possible) for each dam one week after birth. At the age of 3 weeks old, male and female rats in each litter were separately housed.

Blood was collected from the male offspring at 9 and 36 weeks old under anesthetization with ether, and plasma obtained by centrifugation was frozen at –20°C until used in hormone assays. As stated in our previous report[12], only one dam and its pups in the highest dose group survived after delivery, and these pups were only used in the sampling at 36 weeks old. Testes were dissected out and weighed. Testosterone concentrations in plasma were determined with the Wallac Oy kit (Turku, Finland) following the protocols of the supplier. This assay is based on the competition between hormone in a sample and a fixed quantity of labeled hormone for a limited amount of hormone specific antibody. For the assay of testosterone content in testes, part of the organ was homogenized in a glass-teflon homogenizer, and then centrifuged at 700 × g for 10 min at 4°C to remove cell debris and nuclei. The resultant supernatant was used for the assay of testosterone. We also estimated plasma E_2, and the luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations with commercially available kits and protocols (Wallac Oy, and Amaersham, Little Chalfont, UK, respectively). All assays were run in duplicate. Dose response relationships were evaluated by using a one way analysis of variance (ANOVA), followed by Dunnett's test for significant differences between the control and each dosed group. A probability value of $P<0.05$ was considered as significant.

The effects of BPA on the development and growth of the offspring have been presented in our earlier report[12]. No morphological abnormality was observed in the offspring in any of the BPA treatment groups, and the differences in the body weights at 1, 3 and 9 weeks old among groups were not significant. The weight of testes (gram) was 2.78 ± 0.19 (mean ± SD) in the control, and 2.82 ± 0.37 and 2.90 ± 0.25 in 4 mg and 40 mg dose groups, respectively, and the difference either in testes weight or the ratio of testes to body weight (data not shown), was not statistically significant among the groups.

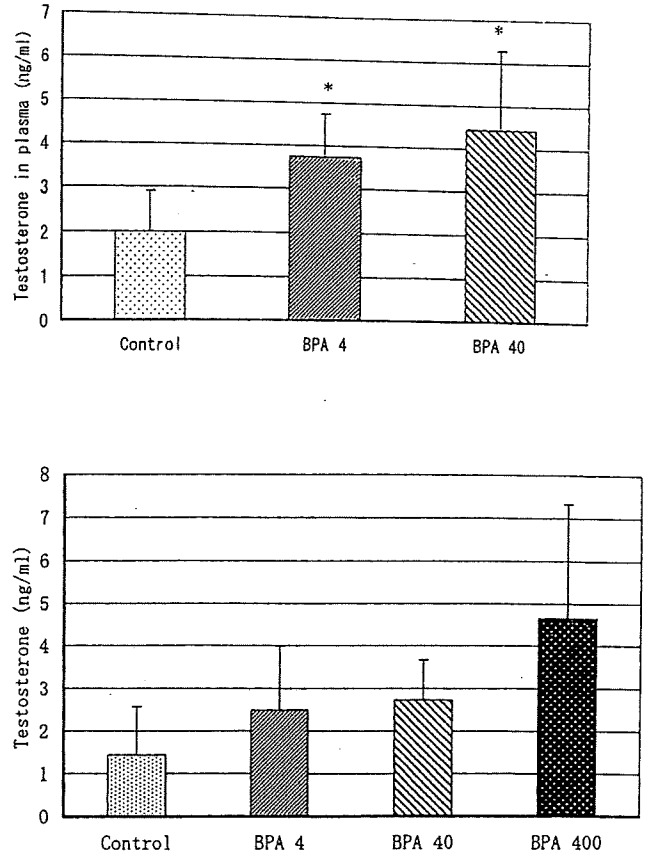

Fig. 1. Plasma testosterone concentrations in male offspring rats at 9 (upper panel) and 36 weeks (lower panel) old.
The bar represents the mean + SD of 4–6 rats in each group. *Significantly different from the control group.

At the age of 9 weeks, the testosterone concentration in the blood of male offspring was 2.00 ± 0.90 (mean ± S.D.) ng/ml in the control group, and it was increased by 88% and 123% in 4 mg/kg and 40 mg/kg BPA groups, respectively (Fig. 1, upper panel). The values in the two BPA groups are significantly higher than that in control group ($P<0.05$). At 36 weeks old, the sex hormone in blood also showed a tendency to increase in a dose-related way in the three BPA groups, although there was no statistical difference between the BPA treatment and control groups (Fig. 1, lower panel).

The secretion of testosterone is regulated by the negative feedback mechanism of the hypothalamus-pituitary-testis axis. Despite the increase in testosterone levels, the LH concentrations in the BPA groups were at the same level as in the control, and the plasma FSH concentrations in the BPA groups also showed little change (Fig. 2). Such an inconsistency in the plasma concentrations of testosterone, LH and FSH was also encountered by other researchers[13]. It is known that part of testosterone is transformed to E_2,

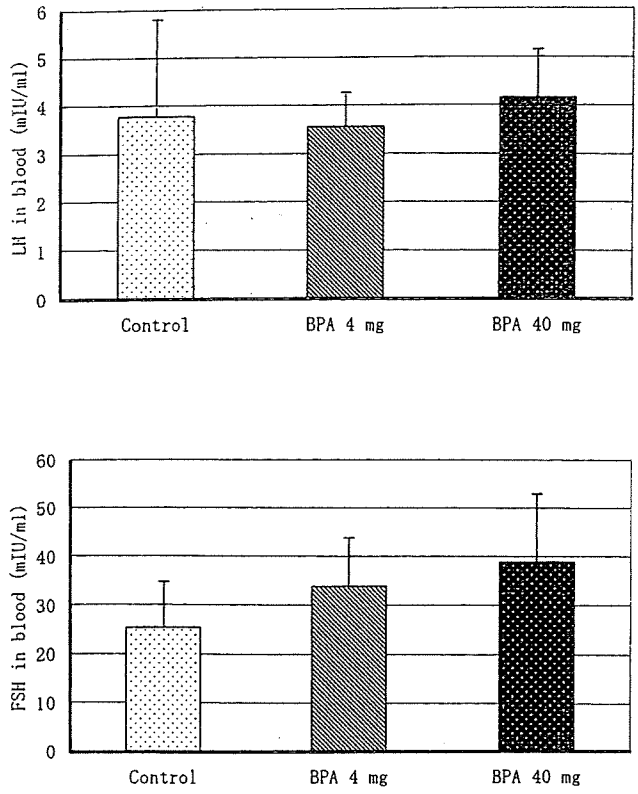

Fig. 2. Plasma LH (top) and FSH (bottom) concentrations in male offspring rats at 9 weeks old.

The bar represents the mean + SD of 5, 4 and 4 rats as the control, and BPA 4 mg and BPA 40 mg groups, respectively.

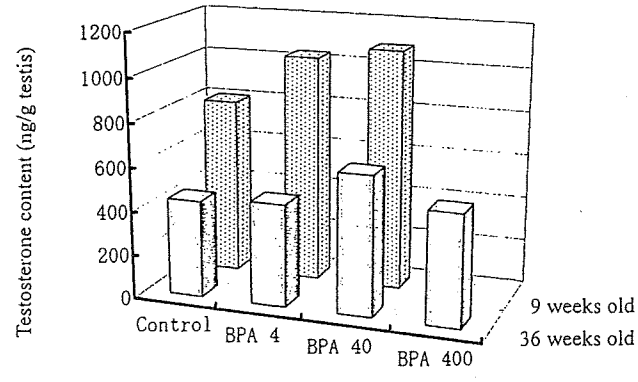

Fig. 3. Testosterone content in testes of the offspring rats at 9 and 36 weeks old.

Each bar represents the mean for 4–6 rats. No significant difference was found between the control and treatment groups at the same age.

and we estimated the plasma E_2 concentrations in the male rats at 9 and 36 weeks old. No significant changes in the plasma E_2 concentration were found in response to BPA administration (data not shown) at either age, indicating that the pathway of E_2 synthesis *de novo* was not affected by this compound.

Testosterone content in testes of male offspring rats at 9 and 36 weeks old was slightly increased in BPA groups in comparison with the control group but the difference was not significant (Fig. 3).

There were few reports about the effects of BPA on sex hormone metabolism in offspring. In this study, we found that blood testosterone level was higher in male offspring of rats perinatally exposed to BPA than in those without the exposure. This effect was most obvious in offspring at puberty (9 weeks), and could still be demonstrated even in adults (36 weeks). This result contrasts strongly with reports from other researchers, in which immature or adult animals were used in most cases. Tohei *et al.* reported that the plasma testosterone concentrations in male rats were decreased when the animals were given BPA subcutaneously as adults[14]. In a most recent report by Saito *et al.*[15], they found a reduction in the blood testosterone level in mice subcutaneously administrated BPA in microgram amounts for 7 weeks in the pubertal period. In other reports, BPA treatment was not shown to have any effect on testosterone formation by cultured Leydig cells from young male rats[16], or the testosterone level in the blood of rats[13, 17]. Nevertheless, in a recent report, Nieminen *et al.* found the plasma testosterone concentrations were increased in field voles treated with BPA[18]. Although there are such discrepancies among the laboratories, which may be attributable to the doses of BPA, the animal species used in the experiments and the age of the animals when treated, it is believed that BPA exposure alters the function and morphology of reproductive organs in the animals directly exposed to it. In regard to the effect of BPA on offspring, this study is the first one, so far as we know, reporting the significant effect on male hormone homeostasis in pups. We did not do histological studies on genital organs in this study, and it is not clear whether there is any pathological change in reproductive organs such as the prostate, which was found to be increased in weight or size in offspring from BPA treated dams[8, 19]. Our results on testes weights are in agreement with other reports[8, 20], in which no change in testes weights was demonstrated despite the noticeable changes in some other genital organs of offspring or adult animals themselves exposed to BPA. The level of testosterone in blood is maintained relatively constant, through a balance between formation mainly in testes and degradation in such tissues as the liver. To elucidate the mechanism(s) underlying the effect of this compound, a study on whether or in which stage the metabolism of testosterone is affected by BPA exposure, is under way.

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Short Communication

Effects of *in Utero* and Lactational Exposure to Bisphenol A on Somatic Growth and Anogenital Distance in F_1 Rat Offspring

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Abstract: Bisphenol A (BPA), a xenoestrogen, has been reported to mimic the actions of estrogen or to affect the endocrine glands *in vivo* and *in vitro*. In this study, we examined whether *in utero* and lactational exposure to BPA altered the somatic growth and anogenital distance (AGD) of F_1 offspring (1, 3, and 9 weeks of age) *in vivo* in rats. Dams were orally administered with various doses of BPA (0, 4, or 40 mg/kg body weight (BW)/day) from gestation day (GD) 6 through postnatal day (PND) 20. There were no significant changes in body weight, liver weight, kidneys weight, testes weight, AGD, the ratio of AGD to BW, or the ratio of AGD to the cube root of BW in BPA exposed pups compared to the vehicle-exposed control. This suggests that prenatal and postnatal exposure (indirect exposure) to BPA (4–40 mg/kg/day, GD 6–PND 20) does not affect on somatic growth or AGD of F_1 generation of male and female rats.

Key words: Bisphenol A, Reproductive toxicity, Body weight, Anogenital distance, F_1 offspring, Rat

Bisphenol A (BPA) is very widely used in the manufacture of polycarbonate and epoxy resins, dental sealants, and other chemical products. BPA released from lacquer coating has been detected in food cans[1], and it has also been found in saliva collected from subjects treated with dental sealants[2]. Krishnan *et al.* have reported weak estrogenic action of BPA eluted from a polycarbonate bottle into medium during the autoclaving procedure. They showed that BPA increased the number of progesterone receptors and promoted the cell proliferation of a cultured cell line which originated from human breast cancer (MCF-7)[3]. BPA induced prolactin (PRL) release *in vitro*[4, 5]. It also increased uterine and pituitary weight, the serum PRL level and the number of immunoreactive PRL cells in ovariectomized Wistar rats[6].

Reproductive toxicity of BPA has been reported in mice and rats. Low-dose effects of BPA *in vivo* were observed in mice. BPA increased prostate and preputial gland weight, and decreased daily sperm production efficiency in male offspring prenatally exposed to BPA at 2 or 20 μg/kg/day from the gestation day (GD) 11 through GD 17[7, 8]. On the other hand, other investigators have failed to find such effects in mice offspring under identical experimental designs[9, 10]. Cagen *et al.* reported that normal reproductive development was observed in offspring born from mothers supplied with BPA in drinking water at a concentration range of 0.01 to 10 ppm (0.001–4.022 mg/kg/day) for 10 weeks, from the premating day (at 9 weeks old) to the weaning day, in Wistar rats[11]. In addition, it was reported that oral high- dose administration (320 mg/kg/day gavage) from GD 11 through postnatal day (PND) 20 resulted in no apparent change in male or female reproductive development in the F_1 offspring of Sprague-Dawley (SD) rats[12].

We determined the effects of BPA exposure from GD 6 through PND 20 on postnatal somatic growth and anogenital

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distance (AGD) in male and female SD rat offspring, because the effects of preweaning exposure on fetus growth and reproductive development have so far remained controversial. Examinations were performed on various clinical and reproductive parameters, including body weight (BW), main organ weight (liver, kidneys and testes), AGD, ratio of AGD to BW (AGD/BW), and ratio of AGD to cube root of BW ($AGD/BW^{1/3}$) at postnatal weeks 1, 3, and 9.

BPA (Bisphenol A standard, purity >99.8%, Cat#: 280-08561, Lot#: HCE9312) and corn oil (Cat#: 034-17015) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. A total of twenty-four pregnant female rats (Crj: CD (SD) IGS strain, 9 weeks of age) at GD 3 were purchased from Charles River Japan Inc. (Tsukuba), separately housed and maintained under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and a 12-h light-dark cycle (0800–2000) throughout the study. The presence of a copulatory plug was considered to be GD 0. A standard laboratory diet (CE-2, CREA Japan, Inc., Tokyo, Japan) and drinking water were available *ad libitum*. Dams were divided into four equal-sized groups (6 pregnant rats/group) randomly, and weighed once a day from GD 3 through PND 20 (except for GD 4–5). The BPA-exposed groups were dosed by oral gavage with 4, 40 or 400 mg/kg BW/day of BPA in corn oil vehicle (10 ml/kg BW), once daily between 0830 and 0930, from GD 6 through PND 20, and the control group was given the same amount of corn oil during the same period. The highest dose, 400 mg/kg, was selected based on a study by Kwon *et al.*[12] reporting no detectable effects of BPA on maternal BW at 320 mg/kg/day from GD 11 through PND 20. The litter size was standardized to ten (male : female = 5 : 5, if possible) between 1000 and 1100 on PND 7 (1 week of age), and then all the culled offspring were used for examination as soon as possible after culling. On PND 21, offspring were weaned and thereafter males and females were housed separately per litter. The developmental parameters of offspring from various dose-treated dams were measured at 1, 3 or 9 weeks after birth. The body weight was recorded with an electric balance (Shimadzu, Kyoto, Japan). The anogenital distance (AGD) (mm) was measured with a digital caliper (Mitutoyo, Kanagawa, Japan) under euthanization by cooling on ice at 1 week or by ether inhalation at 3 or 9 weeks of age in F_1 offspring. AGD/BW (mm/g) and $AGD/BW^{1/3}$ ($mm/g^{1/3}$) were also calculated. A pair of male and female offspring from each dam were dissected at 3 or 9 weeks of age. The liver, kidneys and testes (male) were weighed at 9 weeks of age. Blood collected by decapitation at 1 week of age or sampled from the postcaval vein at 3 or 9 weeks was stored at –20°C for the determination of hormone levels (not reported here). The rest of the offspring were used for investigating behavioral effects (not reported here). The results were expressed as means ± SEM. The differences from the corresponding control group were statistically analyzed by analysis of variance, followed by Dunnett's test ($P<0.05$). The numbers of F_0 and F_1 rats used for examinations in each group are summarized in Table 1. In the control group, 1 female out of 6 was not pregnant. In the 40 mg/kg/day group, all the pups of a dam were found dead on PND 2. In the 400 mg/kg/day group, 4 dams out of 6 died on GD 21. All the pups born from 1 surviving dam in this group were found dead on PND 2. The 400 mg/kg/day BPA group was consequently excluded from further

Table 1. Treatment design and number of subjects examined

Group	Dose (mg/kg/day)	Dams[a]	Live birth[b]	No. of offspring tested			
				Age (wks)	1	3	9
Control	0	6[c]	12.8 ± 0.7	Male	6	5	5
				Female	8	5	5
BPA	4	6	13.5 ± 1.2	Male	9	6	4
				Female	8	6	5
BPA	40	6	11.5 ± 1.1	Male	1	5	4
				Female	10	5	5
BPA	400	6[d]	–	Male	–	–	–
				Female	–	–	–

[a]Six dams per group were dosed BPA or corn oil (10 ml/kg BW) by oral gavage from GD 6 through PND 20.

[b]The number of live offspring per litter on PND 1.

[c]One dam was not pregnant although the presence of a copulatory plug was verified.

[d]Four dams in the 400 mg/kg/day group died during the gestation period (–; Not examined).