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

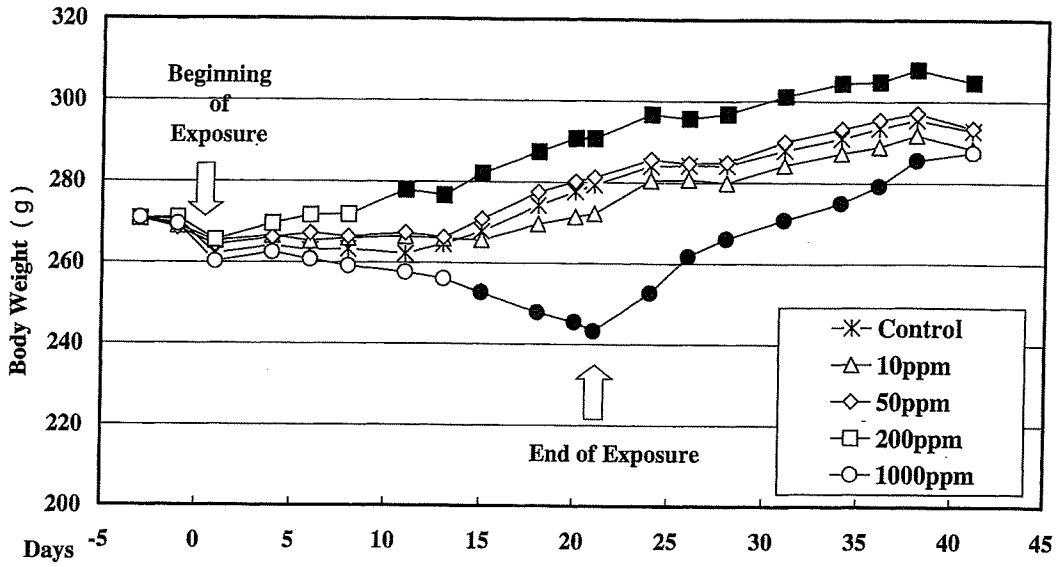

Fig. 1. Time-course changes in body weight of male F344 rats exposed to 1-bromopropane. Mean body weights were determined in each group exposed to 0 (control), 10, 50, 200, and 1000 ppm. Closed marks indicate significant differences from control values at each exposure day at $P < 0.05$ detected with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

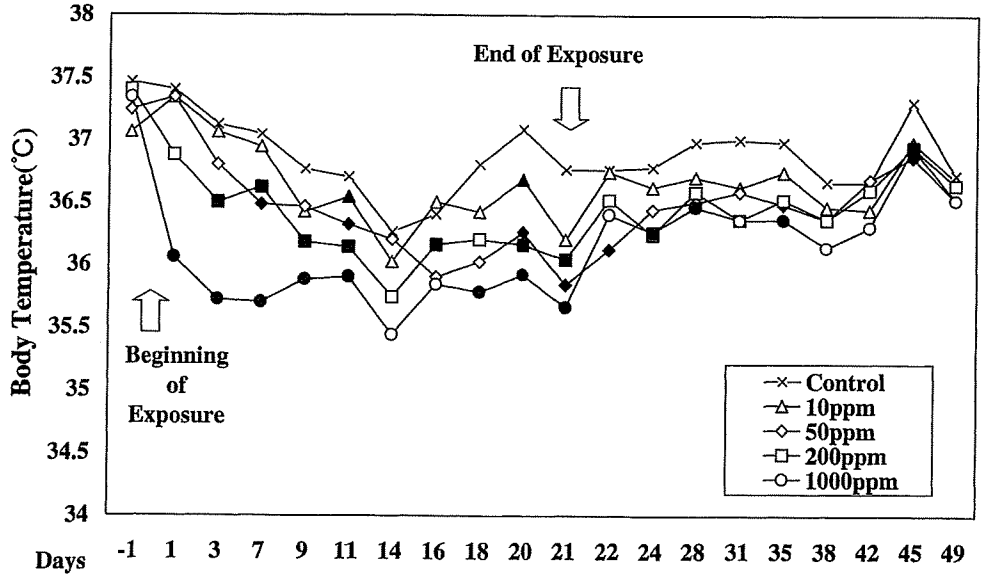

Fig. 2. Time-course changes in body temperature of rats exposed to 1-bromopropane. Closed marks in each exposure group indicate significant difference from control values ($P < 0.05$).

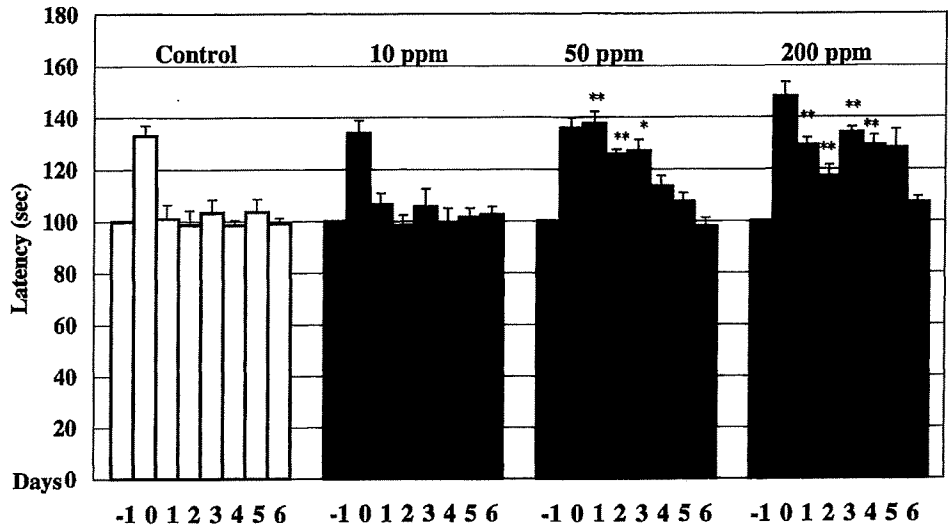

Fig. 3. Effects of 1-bromopropane exposure for 3 weeks on spontaneous locomotor activity of rats during post-exposure dark period. Pre-exposure level was set as 100% for each rat. Mean activity and S.E.M. were calculated from four rats per exposure group and statistical significance of differences from 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

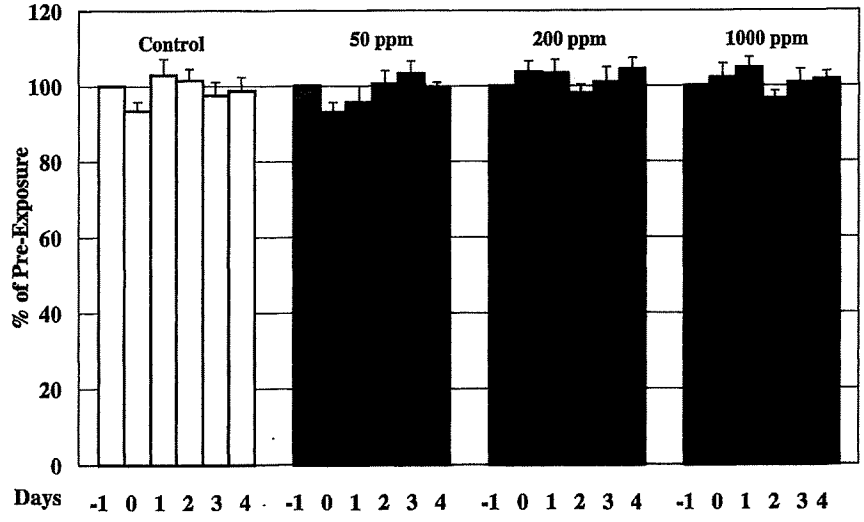

Fig. 4. Effects of a single 8-h dose of 1-bromopropane on spontaneous locomotor activity of rats during post-exposure dark period (see legend to Fig. 3).

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).

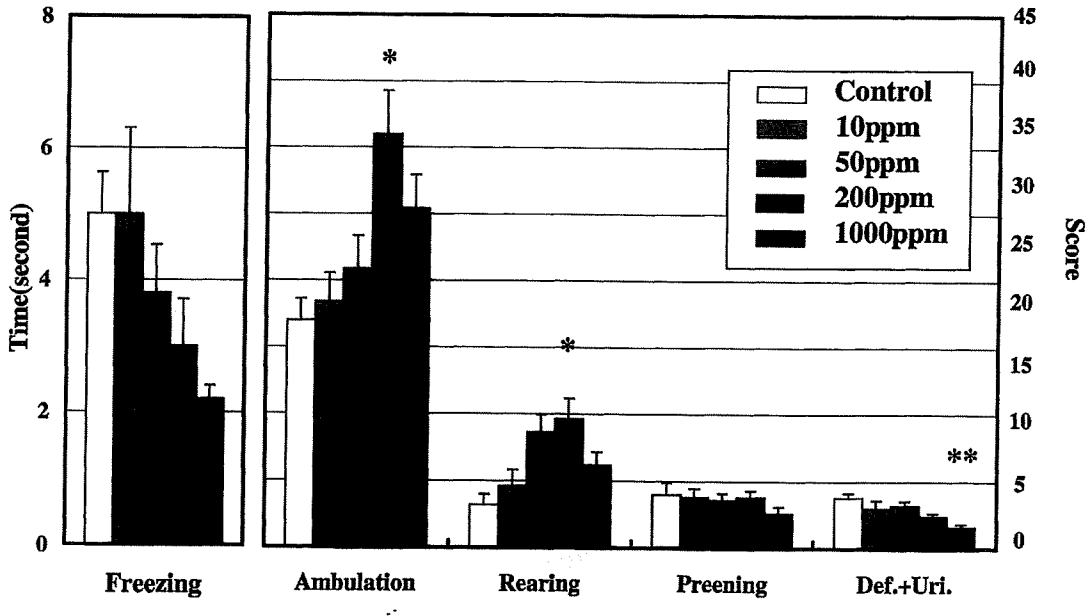

Fig. 5. Effects of 1-bromopropane exposure for 3 weeks on open-field activity of rats. Mean and S.E.M. values of freezing time or behavioral scores were calculated for each exposure group and statistical significance of differences from control was detected with Dunnett's test. Vertical bars indicate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

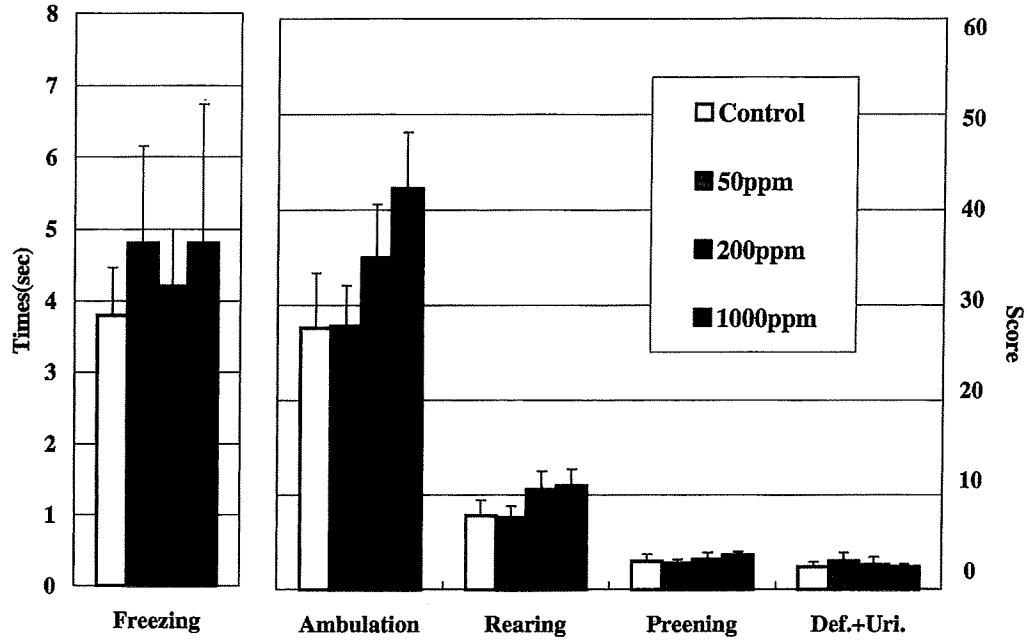

Fig. 6. Effects of a single 8-h dose of 1-bromopropane on open-field activity of rats (see legend to Fig. 5).

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

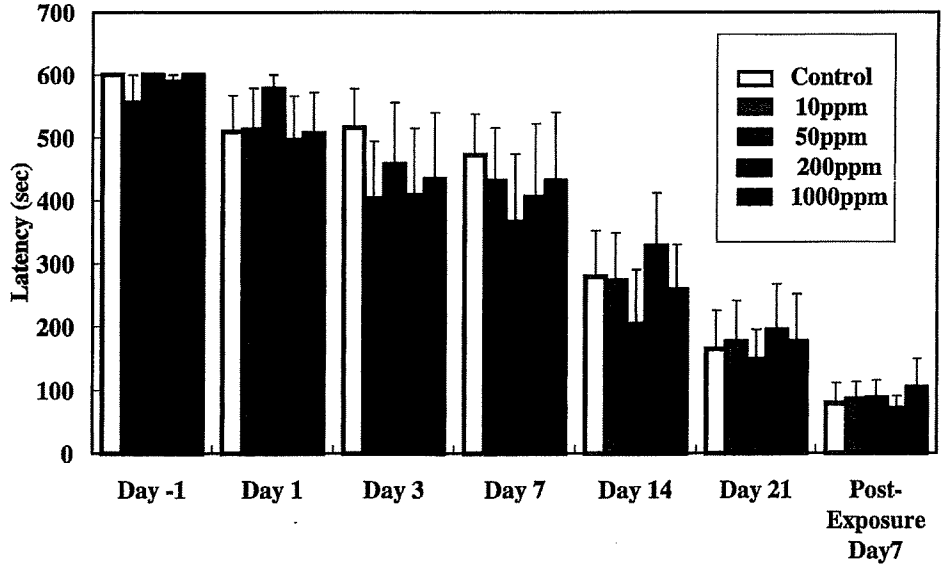

Fig. 7. Effects of 1-bromopropane exposure for 3 weeks on rat passive avoidance. Rats were conditioned to avoid electroshock before exposure and avoidance was tested during and after exposure (pre-exposure conditioning). Mean and S.E.M. values of latency to enter the dark room were calculated for each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

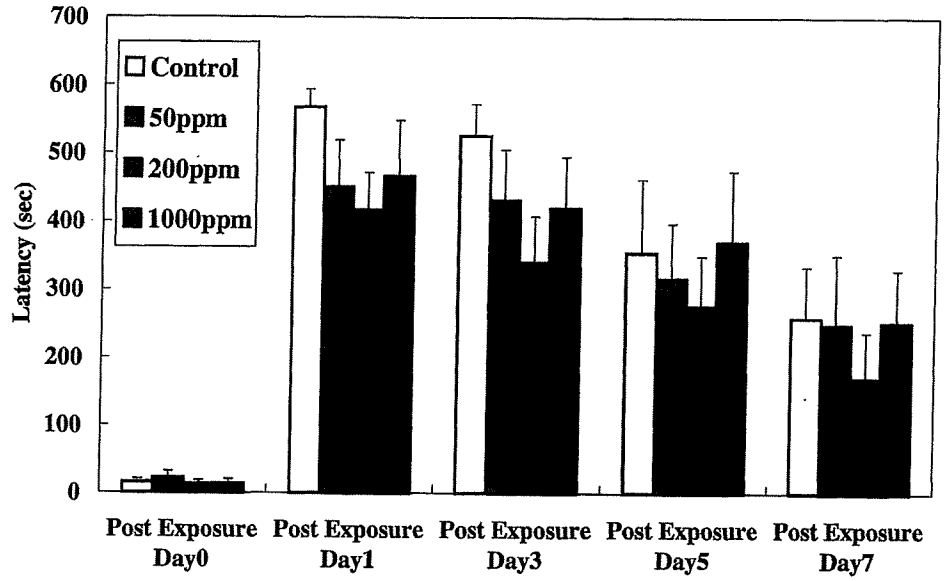

Fig. 8. Effects of 1-bromopropane exposure for 3 weeks on rat passive avoidance. Rats were conditioned after exposure (post-exposure conditioning). Mean and S.E.M. values of latency were calculated for each group.

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

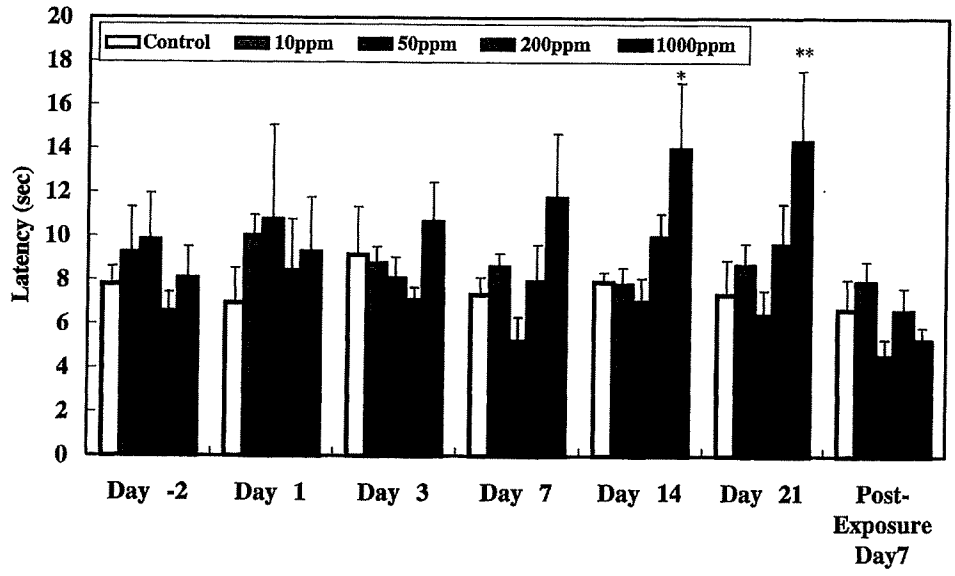

Fig. 9. Effects of exposing rats to 1-bromopropane for 3 weeks on water maze performance. Rats were trained to reach an escape platform before exposure then subjected to water maze testing during and after exposure (pre-exposure learning). Mean and S.E.M. values of latency required to reach the escape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

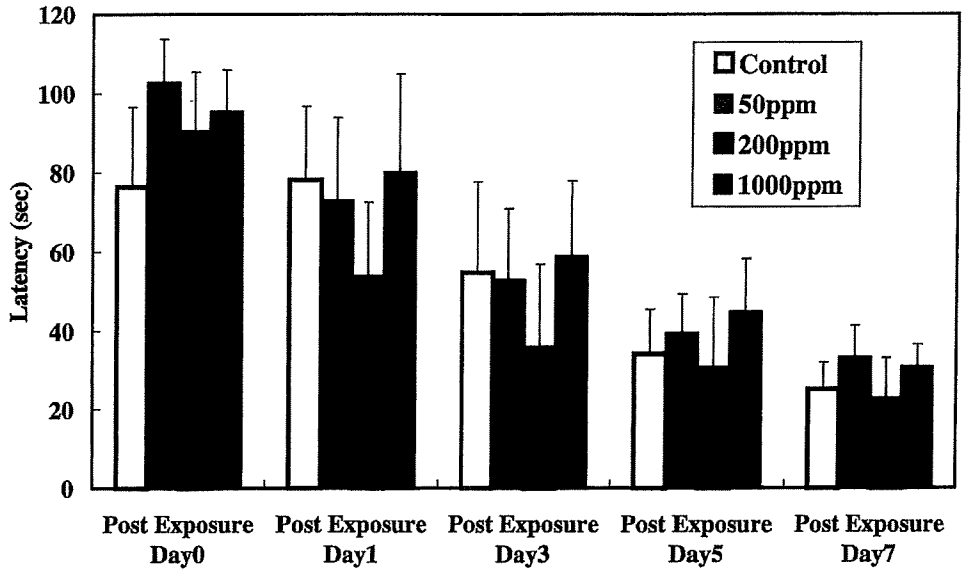

Fig. 10. Effects of exposing rats to 1-bromopropane for 3 weeks on water maze performance. Rats were trained to reach an escape platform after exposure (post-exposure learning) (see legend to Fig. 9).

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

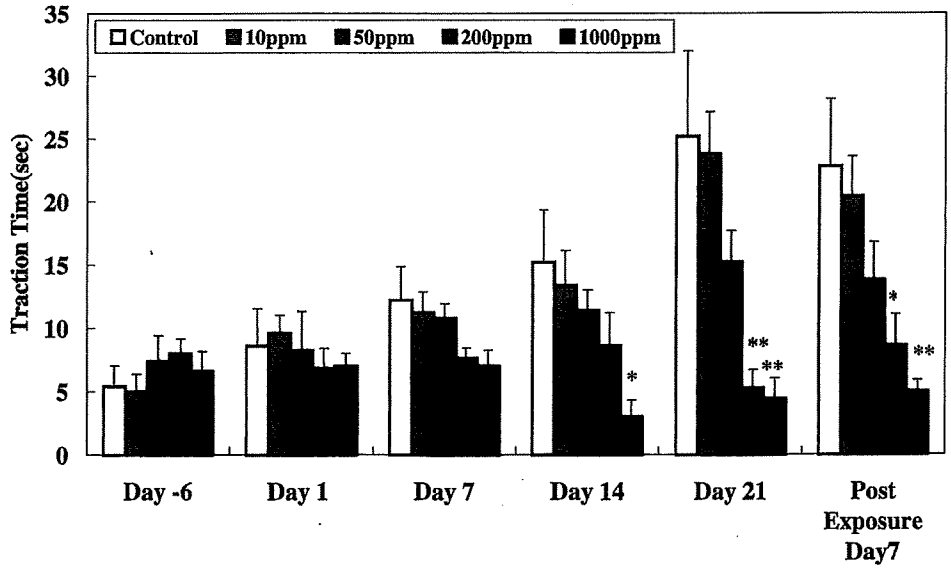

Fig. 11. Effects of exposing rats to 1-bromopropane for 3 weeks on traction time. Mean and S.E.M. values of traction time were calculated for each exposure group and statistical significance of differences from control was detected with Dunnett's test. (*) $P < 0.05$; (**) $P < 0.01$.

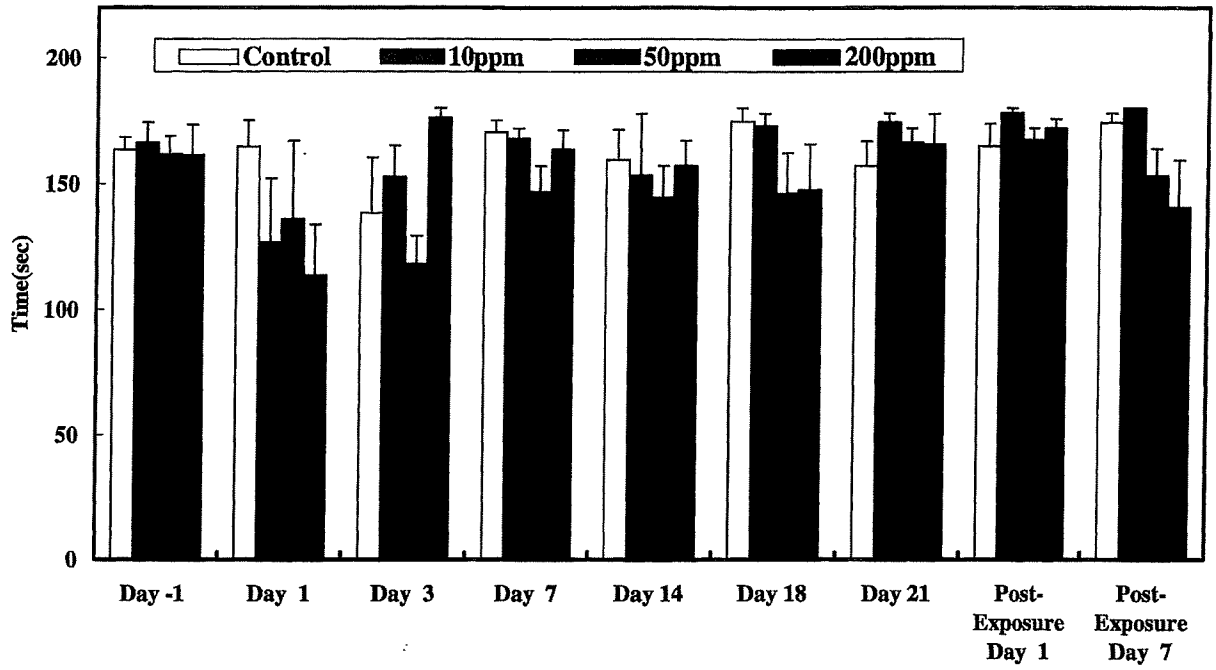

Fig. 12. Effects of exposing rats to 1-bromopropane for 3 weeks on rota-rod performance. Mean and S.E.M. values of time remaining on a rotating rod were calculated for each group.

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.

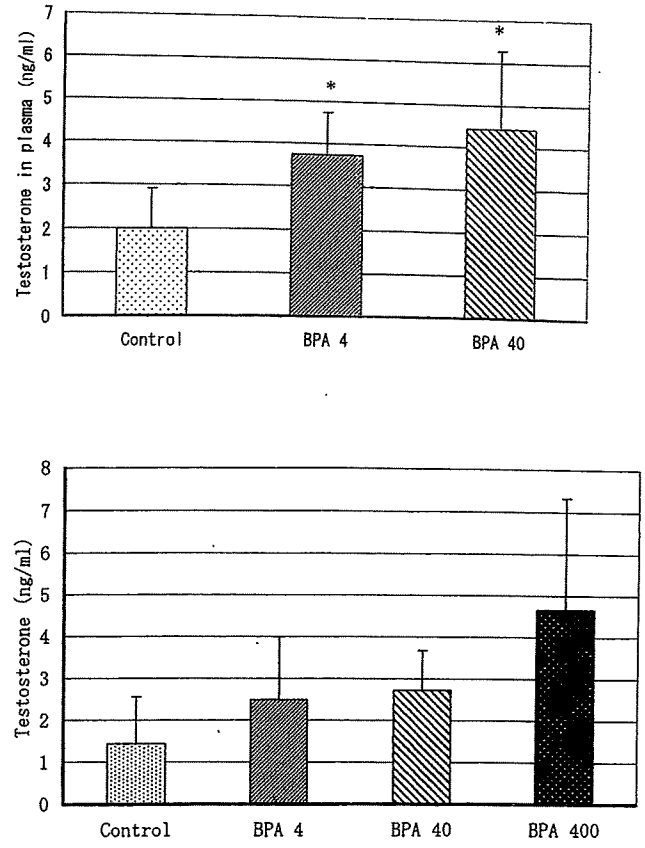

Fig. 1. Plasma testosterone concentrations in male offspring rats at 9 (upper panel) and 36 weeks (lower panel) old.
The bar represents the mean + SD of 4–6 rats in each group. *Significantly different from the control group.

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,

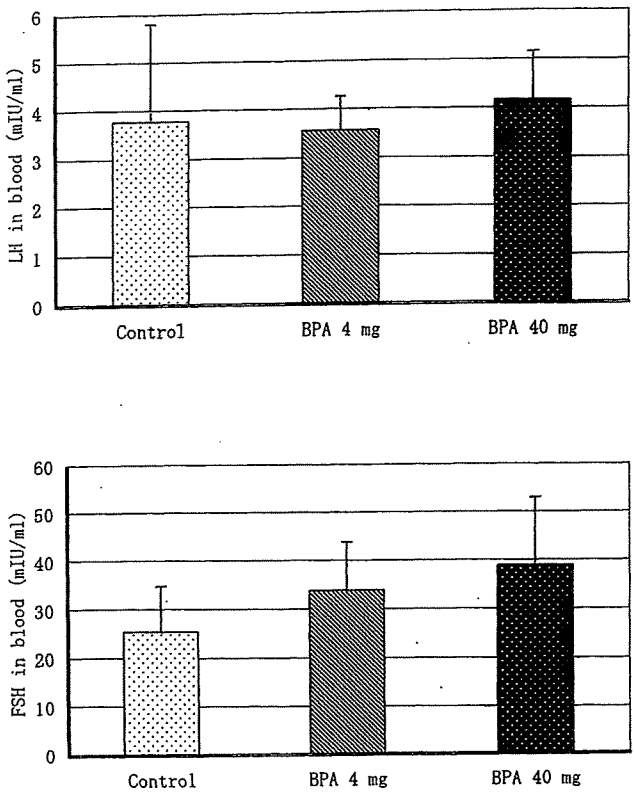

Fig. 2. Plasma LH (top) and FSH (bottom) concentrations in male offspring rats at 9 weeks old.

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.

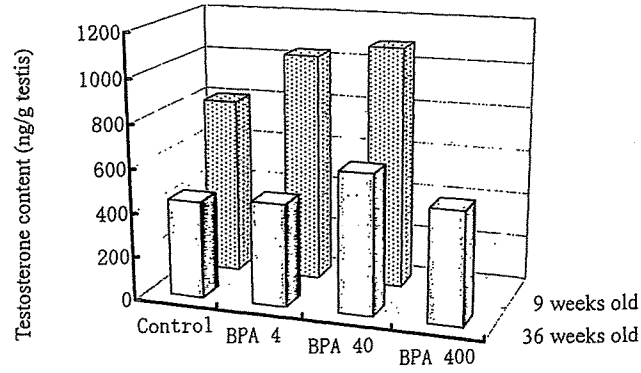

Fig. 3. Testosterone content in testes of the offspring rats at 9 and 36 weeks old.

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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Review

Toxicologic/carcinogenic Effects of Endocrine Disrupting Chemicals on the Female Genital Organs of Rodents

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Abstract: Toxicologic/carcinogenic effects of some representative endocrine disrupting chemicals (EDCs) having estrogenic activity, such as alkylphenols, on the female genital organs of rodents, especially rats, are reviewed and discussed, focusing on our recent research. Neonatal treatment of high-dose p-tert octylphenol (t-OP, 100 mg/kg s.c. injection every other day from postnatal day 1 (PND 1) to PND 15) induced various long-term persistent irreversible effects on the female reproductive system of Donryu rats, such as lower gonadotropin levels at prepuberty, inhibition of uterine gland genesis, persistent estrus and polycystic ovaries. The result indicates that neonatal high-dose treatment of estrogenic EDCs can affect gonadotropin secretion during the developmental period of sexual maturation with direct masculinization of the hypothalamic function. Exposure limited to the first 5 days after birth (PNDs 1–5) to 100 mg/kg t-OP, however, caused delayed influence which was characterized by accerelated appearance of atrophic ovary, manifested by early-occurring and long-term continuing persistent estrus after puberty, whereas no abnormalities could be found with regard to growth and differentiation of the reproductive organs and the hypothalamo-pituitary-gonadal control system up to maturation, the influence being caused by delayed modulation of the hypothalamo-pituitary-gonadal control system. The most notable effect on the female reproductive system when normal cycling rats were exposed to high-doses t-OP for 28 days, was disappearance of the estrous cycle, but no clear changes were detected in other parameters such as uterine weight and morphology. These results indicate that the most serious issue with EDCs is the potential effects of prenatal and/or neonatal exposure on rodents. Well or moderately differentiated adenocarcinomas increased in Donryu rats initiated with N-ethyl-N'-nitro-N-nitrosoguanidine, when high-dose t-OP was given subcutaneously during adulthood. Neonatal exposure for PNDs 1–5 to high-dose t-OP also showed promoting effects on uterine adenocarcinoma development. However, in rats given t-OP for PNDs 1–15, uterine tumor malignancy was clearly increased, although there was no significant alteration in the total incidence of adenocarcinomas. The results are very interesting in consideration of the histogenesis of uterine adenocarcinomas. However, maternal exposure to low doses of EDCs such as nonylphenol and bisphenol A at actual human exposure levels by the oral route showed no effects on growth and development of the female reproductive system or uterine carcinogenesis. These results indicate that dietary exposure to low doses of EDCs might not induce any adverse effects on the female genital system in mammals including humans. (J Toxicol Pathol 2004; **17**: 69–83)

Key words: endocrine disrupting chemicals (EDCs), toxicity/carcinogenicity, female genital organs, rodents

Introduction

Recently, the possible adverse consequences arising from the release of man-made substances with estrogenic, anti-estrogenic or androgenic properties, so called endocrin disrupting chemicals (EDCs), into the environment have become an important environmental problem. There is much concern that these EDCs may have the potential to disturb normal sexual differentiation and development in wild life and mammals, including humans, and exert various deleterious effects on many organs, with carcinogenic effects being particularly important in mammals. The genital organs are the obvious target organs of various EDCs, and various toxicologic changes have been reported to be induced in both male and female genital organs of rodents. Unfortunately, however, there is less information

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on females than males, although many EDCs have estrogenic properties.

In the present report, toxicologic/carcinogenic effects of some representative EDCs, such as alkylphenols, on the female genital organs of rodents, especially rats, are briefly reviewed and discussed from the point of view of extrapolation to humans, mainly focusing on our recent research. In our studies of the effects of EDCs, Donryu rats were mainly used. The Donryu rat is a unique domestic strain having a regular 4-day estrous cycle at the juvenile stage. After 5 months of age, however, persistent estrus appears and increases age-dependently, and a high occurrence of spontaneous uterine adenocarcinomas is observed at about 2 years of age or thereafter (Table 1)[1]. In this rat strain, the early appearance of persistent estrus results in an increase in the estrogen (E2):progesterone(P) ratio (E2:P ratio). In humans, it has been reported that relatively high E2:P values increase the endometrial cancer risk[2,3]. Using this strain, we recently demonstrated effects of reproduction on uterine carcinogenesis, in line with the known lower risk of uterine adenocarcinoma in multiparous as compared to nulliparous or infertile women. The incidence of spontaneous endometrial adenocarcinomas showed a tendency to decrease in animals having three reproductive experiences, compared to the nulliparous case, although the incidence was not influenced by a single pregnancy[4]. Thus, this rat strain is a good animal model for endometrial adenocarcinoma development due to the imbalance of endogenous steroid hormones, as found in humans. We also succeeded in obtaining high induction of tumors in this strain by single intra-uterine administration of N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), thereby establishing a two-stage uterine carcinogenesis model (Fig. 1)[1,5]. Quite recently, Vollmer[6] reviewed experimental endometrial cancer models, including Donryu rats, and considered them useful for studies on molecular aspects of endometrial cancer and carcinogenesis.

Table 1. Persistent Estrus and Spontaneous Uterine Tumors in Donryu and F344 Rats*

Sequential Changes in Persistent Estrus Incidences in Female Donryu and F344 Rats

	Incidence (%)						
Strain	4	5	6	8	10	12	15 (Months of age)
Donryu	0	17	32	64	87	88	85
F344	0	0	2	0	6	11	4

Main Spontaneous Uterine Tumors in Donryu and F344 Rats

Uterine tumors	Incidences (%)	
	Donryu	F344
Mean survival time (weeks)	108.8 (62–120)	114.1 (60–131)
Endometrial adenocarcinoma	35	1
Endometrial stromal polyp	1	21

*: Maekawa *et al.*, J Toxicol Pathol 1999; 12: 1–11.

Classification of EDCs

Various chemicals have been shown to have endocrine disrupting effects not only on wildlife but also mammals including humans. Major representative EDCs are as follows, according to their use, chemical structures and/or chemical characteristics.

1. DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane), DDE (1,1'-(dichloro-ethenylidene)bis(4-chlorobenzene)), dieldrin: agricultural chemicals (insecticides) with properties of high-accumulation and resistance to degradation.

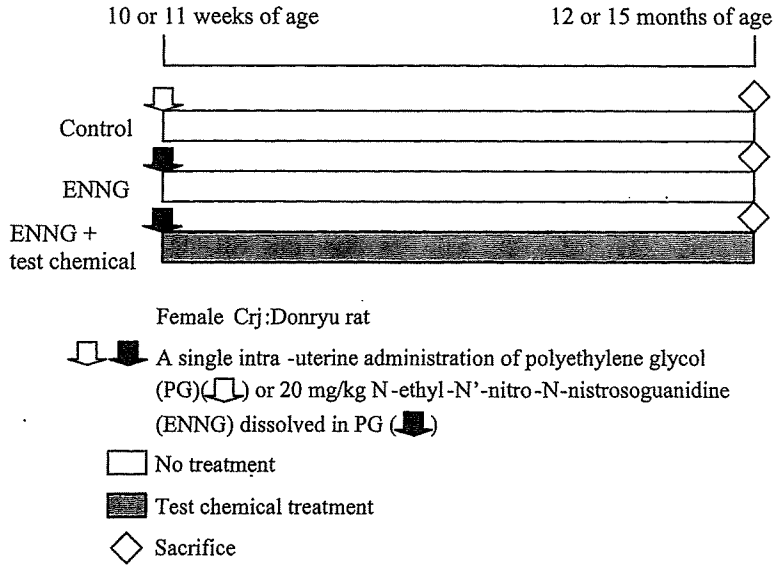

Fig. 1. Two-stage rat uterine carcinogenesis model

2. PCBs (polychlorinated biphenyl), PBB (polybrominated biphenyl): industrial chemicals (insulators etc.) which accumulate and are difficult to degrade.
3. DEHP (di(2-ethylhexyl)phthalate), alkylphenols such as nonylphenol and octylphenol, bisphenol A: industrial chemicals widely used as plasticizers or surfactants.
4. Dioxins such as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), dibenzofuran: chemicals naturally produced by dust-incineration.
5. TBT (tributyltin), TBTO (tributyltin oxide): industrial chemicals used for coating of ships' bottom.
6. DBCP (1,2-dibromo-3-chloropropane), atrazine, vinclozolin: agricultural chemicals.
7. DES (diethylstilbestrol), tamoxifen, oral contraceptives: medical drugs.

Development of the Female Genital Organs and Profile of Hormonal Secretions in Rodents

In general, development of the female genital organs in rats is roughly classified into 3 stages, prenatal (embryonic), neonatal/juvenile and adult/aged. The prenatal stage is from the day of fertilization, i.e., day 1 post-coitum till birth (about 20–22 days in rats). The embryonic bipotential gonad develops from mesoderm in the gonadal ridge located on the dorsal coelomic walls. The primordial germ cells and the gonadal ridge are visualized as condensations of cells localized ventral to the mesonephrons by gestational day 12 in rodents. In the embryonic developmental stage, two sets of paired tubular organs develop: the Wolffian ducts and the Mullerian ducts. It is well established that the presence or absence of functioning embryonic testes plays a major role in determining which duct system undergoes further development. In the rat, the critical time period for Mullerian duct development covers days 14–18 of gestation. On day 18, in the absence of testicular hormones including anti-Mullerian hormone (AMH) from Sertoli cells, the Mullerian ducts undergo further development and the Wolffian ducts degenerate.

The female reproductive tract of rodents is immature at birth and the developing uterus undergoes a period of rapid growth and differentiation during the first 2 weeks of postnatal life. In rats, the uterus at birth corresponds developmentally to the fetal uterus at gestation day 100 in human beings[7]. During this period, luminal epithelial cells invaginate into the underlying stroma to form uterine glands[8]. The uterine growth phase in this period coincides with an elevation of serum estradiol levels beginning on postnatal day (PND) 9. Thus, the role of endogenous estrogen (17β-estradiol, E2) and its receptor (ER) are very important for uterine growth and differentiation. In normal rats, ER expression in the uterine epithelium appears at various days from PND 7 to PND 15[9].

In female rats, serum FSH (follicle-stimulating hormone) levels rise to a peak at PNDs 15–16 followed by an abrupt nadir, while LH (luteinizing hormone) concentrations are high at PNDs 2–10 followed by gradual decline during sexual maturation; E2 levels also rise to a peak during the first 2 weeks of age[10,11]. On the other hand, α-fetoprotein, the estrogen-binding protein produced in the liver, is found in very high concentrations for several weeks after birth[12]. It is well known that the increase of FSH before puberty is caused by nullification of the negative feedback of estrogen because of the high concentrations of serum α-fetoprotein[13].

A striking sexual dimorphism in gross morphology of the medial preoptic area (sexually dimorphic nucleus of the preoptic area: SDN-POA) has been recognized in the rat brain[14]. The development of this nucleus starts during late fetal life and depends on the hormonal environment at the critical period of sexual differentiation[14–16]. In genetic males, the relatively high levels of perinatal testosterone are aromatized to estradiol in the nervous cells of SDN-POA and the estrogenic signals may be directly responsible for the increase of SDN-POA volume. In genetic females, in contrast, estrogenic effects on SDN-POA are prevented because estrogen is bound to the serum binding protein, α-fetoprotein, during the late embryonic and early neonatal periods. The female SDN-POA is smaller than that of males as a result of an orchestrated pattern of decreased cell proliferation and/or increased programmed cell death[17,18]. It is well known that the SDN-POA volume of genetic females becomes larger than normal on perinatal exposure to testosterone or high amounts of some estrogenic compounds[19]. Analogues of SDN-POA have also been identified in various animal species such as the gerbil, Guinea pig, ferret, quail and human[20]. Recently, another sexual dimorphism had been demonstrated in the anteroventral periventricular nucleus of the preoptic area (AVPvN-POA) and the locus coeruleus[21,22]. The volumes of these are larger in females than males, but a direct correlation with the hormonal environment has not yet been clarified.

In the rat brain, ERα expression is found primarily in ventral midline structures such as bed nucleus of the stria terminals, hypothalamic medial preoptic area, hypothalamic ventromedial nucleus, hypothalamic arcuate nucleus, septohypothalamic nucleus, septum and central gray area of the midbrain. ERβ is similarly distributed in the brain and is additionally detected in the paraventricular nucleus of the hypothalamus and the hippocampus[23,24].

After weaning, the first estrous cycle starts at about 36 days of age, and the minimum breeding age is about 84 days of age[25]. The estrous cycle is characterized by cyclic changes of the epithelial surface in the vagina and the uterus. The estrous cycle in the rat lasts 4–5 days and is divided into proestrus, estrus, metestrus and diestrus. Uterine weights increase with luminal excretion from diestrus to proestrus, showing a peak at proestrus, but decrease at estrus and metestrus.

In normal cycling rats, the E2 and P levels are highest at proestrus, corresponding with the increased uterine weights. Thereafter, the E2 level drops toward estrus and slightly increases again at diestrus. The P value increases slightly at metestrus, although it is low at estrus and diestrus. At

proestrus and diestrus, and especially the former, the luminal and glandular epithelial cells along with stromal cells beneath the luminal epithelium are strongly positive for ER-alpha mRNA expression. At estrus, the expression is slightly diminished in luminal cells, but is almost completely lacking in glandular cells. At metestrus, positive signals appear again in the latter. In the myometrium, expression is constant in all estrous stages. Thus cell-type specific patterns of ER-mRNA expression characterize the uteri of normal estrous cycling rats[26].

In general, the adult stage in rats is from minimum breeding age to maximum age (about 360–450 days), and thereafter the aged stage starts[25]. In Donryu rats, however, estrous cycle abnormalities increase age-dependently after 26 weeks of age, and almost all animals show persistent estrus at 52 weeks of age. In contrast, vaginal smears of F344 rats indicate a normal estrous cycle until 52 weeks of age[27]. In our studies, various histological changes such as follicular cysts and atrophic changes such as absence of corpora lutea in the ovary and cornification of epithelium in the vagina in Donryu rats were observed to be linked with persistent estrus, and increased with time, especially after 10 months of age. In F344 rats, in contrast, atrophy of the ovary is observed in only a few animals at 15 months of age. As a result of ovarian atrophy, in Donryu rats, the plasma values of E2 and P, and especially the latter, decrease with age, the E2:P ratio becoming elevated, and the bromodeoxyuridine (BrdU)-labeling indices of uterine endometrial cells are age-dependently increased[28]. This age-related hormonal imbalance and the constant high level of proliferating activity of epithelial cells are considered to play important roles in high yield development of spontaneous uterine endometrial adenocarcinomas in this rat strain[1,28].

Toxicologic and/or Carcinogenic Effects of EDCs on the Female Genital Organs of Rodents

Rodents in the first 2 weeks of postnatal life, termed "a critical point" or "a window of vulnerability", are very sensitive to exogenous estrogens and androgens including EDCs, because the reproductive tract undergoes rapid growth and differentiation within this period, as mentioned above. Thus, the OECD (Organization for Economic Cooperation and Development) recently proposed the immature rat uterotropic assay as one of the screening test methods for the detection of estrogenic or anti-estrogenic properties of chemicals[29]. In studies using adult animals, the ovariectomized (OVX) animal model is also effective for the detection of estrogen agonists, because the effects of endogenous estrogen can be minimized[30]. In various toxicity studies using adult animals, oral administration has generally been used to assess the toxicity of chemicals, and the OECD has also proposed a new 28-day repeated oral-dosing toxicity test protocol using adult rats, the enhanced OECD TG407 protocol, for the assessment of the toxic effects of EDCs. For the detection of endocrine disrupting activity of direct-acting chemicals, however, other administration routes such as subcutaneous injection may provide greater sensitivity than oral administration, because this eliminates the direct effects of metabolism of the chemicals during first passage through the liver.

As mechanisms for the biological effects of EDCs on their target organs, their binding to growth factor receptors and arylhydrocarbon (Ah) receptors as well as steroid receptors has been considered to be very important. Furthermore, some chemicals have been shown to have effects on endogenous estrogen metabolism, resulting in disturbance of the hormonal milieu.

Effects of High-doses of EDCs

Effects on Growth and Development of Female Reproductive Organs

Prenatal and/or Neonatal Exposure

Inappropriate exposure to estrogens and also EDCs in the prenatal and/or neonatal period has been well established to exert irreversible influence directly and indirectly on the female reproductive system[31,32]. "Androgenization" is characterized by direct modulation of the hypothalamo-pituitary-gonadal control system, resulting in lowering of gonadotropin levels and persistent estrus as an indirect effect, and abnormal uterine/vaginal development and/or growth as direct influences.

Alkylphenolic compounds are derived from biodegradation of nonionic surfactants, alkylphenol ethoxylates, which are widely used as detergents in many industries. Alkylphenol ethoxylates are also broken down in the process of sewage-treatment or in rivers into alkylphenols, such as nonyl or octylphenol (NP or OP), which are well known representative EDCs with weak estrogenic activity, acting via binding to ER. In vitvo data indicate that OP has the most potent estrogenic activity of the alkylphenols (approximately 1000 times less estrogenic than E2), although NP is detected with higher levels than OP in the environment.

In our studies of the toxicologic/carcinogenic effects of EDCs on the female genital organs, OP was selected as a representative compound. It has already been reported that neonatal treatment with OP disrupts estrous cyclicity after weaning in female rats[33]. We also examined the effects of neonatal exposure to a high dose of p-tert octylphenol (t-OP) on the female genital organs of Donryu rats[34], and the results were in line with those of other papers: long-term persistent irreversible effects such as lower gonadotropin levels at prepuberty, inhibition of uterine gland genesis, persistent estrus shown by vaginal cytology, and polycystic ovaries. In our recent study, newborn female pups were injected with 100 mg/kg t-OP subcutaneously within 24 h of birth. Administration was repeated every other day until PND 15 (PNDs 1–15), and animals were observed till PND 77. Histologically, inhibition of uterine gland genesis was apparent during the immature period before weaning. The day of vaginal opening was about 4 days earlier in OP-treated animals than in controls, and after vaginal opening,

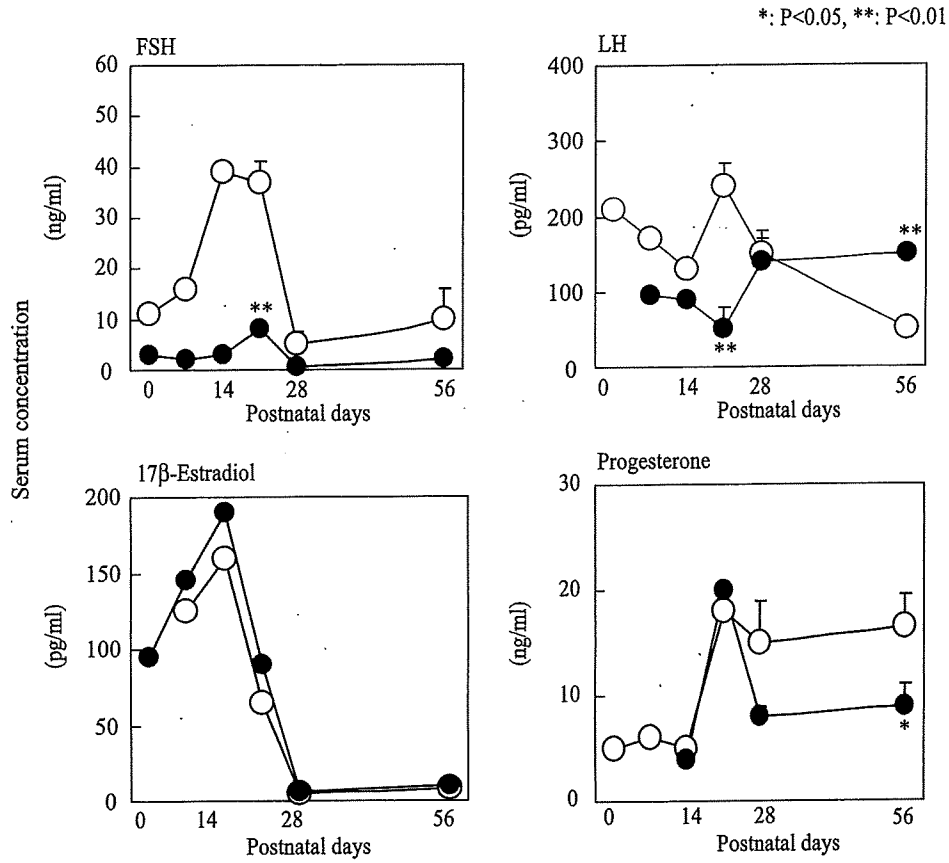

Fig. 2. Serem gonadotropins and sex steroid hormones in control and PNDs 1–15 OP-treated rats. Open circles (○), controls; black circles (●), PNDs 1–15 OP-treated. Katsuda *et al.*, Toxicol Appl Pharmacol 2000; 165: 217–226.

none of the OP-treated rats showed a regular estrous cycle, and persistent estrus was ultimately observed in these animals. Atrophic and polycystic ovaries without corpora lutea were anovular. In the endometrium, cell-proliferative activity and cell-death were increased and decreased, respectively, and expression of estrogen receptor alpha mRNA was apparent on in situ hybridization. At 8 weeks of age, treated animals exhibited luminal epithelial hyperplasia with overexpression of ER-mRNA. During the immature period, serum FSH and LH levels were consistently lower in OP-treated rats than in controls. In particular, serum FSH levels remained uniformly low. Serum E2 levels demonstrated essentially the same pattern as in controls, being elevated at PND 14, and then falling to low levels. After weaning before sexual maturation, FSH values in treated rats remained low, while those of control animals decreased rapidly and were maintained at the same levels as in the OP-treated case. In contrast, LH levels of treated animals increased after weaning and remained high until the end of the experiment (PND 77). Serum P levels of both OP-treated and control rats were constant, but the level in the former was only half of the latter value (Fig. 2). Serum inhibin levels of OP-treated rats were nearly the same as in controls at PND 28. The results resembled those of male or androgenized female rats in the secretary pattern of gonadotropins at this age[10,11], indicating that neonatal treatment with high-dose t-OP affects gonadotropin secretion during the developmental period of sexual maturation with direct masculinization of hypothalamic function.

In another of our studies, neonatal exposure for the first 2 weeks (PNDs 1–15) to 100 mg/kg t-OP induced an early and enhanced ER expression in the luminal epithelium compared with age-matched controls, and increased proliferating cell nuclear antigen (PCNA) positive cells, though expression in the glandular epithelium was suppressed in relation to inhibited gland-genesis. Therefore neonatal exposure to high doses of EDCs with estrogenic activity can induce abnormal differentiation in the developing rat uteri via abnormal ER expression and subsequent alteration of cell proliferating activity[35].

Recently, however, it has been reported that prenatal and/or neonatal exposure to high doses of estrogens or EDCs with estrogenic activity also exerts a "delayed" influence, different from that of typical androgenization. The delayed influence is probably caused by delayed modulation of the hypothalamo-pituitary-ovarian control system[36]. A number of investigators have described effects of neonatal exposure