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Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice

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Abstract

Bisphenol-A (BPA), one of the most common environmental endocrine disrupters, has been extensively evaluated for toxicity and carcinogenicity. However, little is still known about its action on the CNS. Here we found that prenatal and neonatal exposure to BPA resulted in the enhancement of the rewarding effect and hyperlocomotion induced by morphine in mice. Under these conditions, no change in the G-protein activation by morphine and μ-opioid receptor expression in the lower midbrain was observed by prenatal and neonatal exposure to BPA. These results suggest that chronic exposure to BPA produces the supersensitivity of the morphine-induced rewarding effect and hyperlocomotion without direct changes in μ-opioid receptor function in the lower midbrain. The present data provide further evidence that prenatal and neonatal exposure to BPA can directly influence the development of the central dopaminergic system.

Keywords: Bisphenol-A; Morphine; Rewarding effect; Hyperlocomotion; μ-Opioid receptor; G-Protein activation

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. It has been reported that administration of bisphenol-A (BPA) to pregnant mice on gestation days at a dose that is within the range typical of the environmental exposure of humans produces significant changes in the postnatal growth rate and brings on early puberty in these mice [6]. These results imply that BPA may cause unpleasant toxicity in the developmental process. In contrast, little is known about the neuronal toxicity induced by BPA in the CNS. More recently, we have reported that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced rewarding effect and central dopamine D_1 receptor function in mice [19].

Many studies have suggested that the mesolimbic dopaminergic system which projects from the ventral tegmental area (VTA) to the nucleus accumbens is critical for the initiation of opioid reinforcement and hyperlocomotion [7,16,20]. Either DAMGO- or morphine-induced place preference can be blocked by dopamine antagonists receptor [13,14]. As well as the rewarding effect, it has been well recognized that hyperlocomotion induced by morphine can be blocked by treatment with dopamine receptor antagonists in the nucleus accumbens [4,8]. These findings indicate that the dopamine-containing neurons of the midbrain VTA, which has a high density of μ-opioid receptors, play a critical role in the rewarding effects and hyperlocomotion by μ-opioids. In terms of dopamine receptor involvement at the terminal site of the mesolimbic dopamine system, the rewarding effect of abused drug has been shown to be mediated by dopamine D_1 receptors [10,14,17], which could be directly affected by prenatal and neonatal exposure to BPA [19]. The aim of the present study was to investigate whether prenatal and neonatal exposure to BPA in mice could affect the rewarding and locomotor-enhancing effects induced by morphine.

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. All efforts were made to minimize the number of animals used and their suffering. All experiments were performed using male ddY mice that had been prenatally and neonatally exposed to BPA. Prenatal and neonatal BPA exposure was conducted as previously described [19]. Adult female mice were chronically treated with BPA-admixed powder food containing 0 (B0), 0.002

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(B0.002), 0.5 (B0.5) and 2 (B2) mg BPA/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of BPA from their mothers. During the treatment with BPA, animals did not show weight loss and disrupted maternal behaviors.

Place conditioning was conducted as previously described [18]. The apparatus was a shuttle box (15 × 30 × 15 cm: w × l × h) which was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor, and the other was black with a smooth floor to create equally preferred compartments. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injections. The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. A day after these conditioning sessions, the animal was placed in the test apparatus without any confinements.

The locomotor activity of mice was measured by an ambulometer as described previously [11]. Briefly, a mouse was placed in a tilting-type round activity cage 20 cm in diameter and 19 cm high. Any slight tilt of the activity cage, which was caused by horizontal movement of the mouse, was detected by three microswitches. Total activity counts were automatically recorded for 3 h following the injection of saline (10 ml/kg, s.c.) or morphine (10 mg/kg, s.c.). Total activity was counted for 3 h after the treatment.

In the membrane preparation, mice were killed by decapitation and the lower midbrain was then dissected as described previously [12]. The lower midbrain was rapidly excised at 4 °C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris–Mg^{2+} buffer containing 50 mM Tris–HCl (pH 7.4), $MgCl_2$ and 1 mM EGTA for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4 °C for 10 min at 48,000 × *g*. The pellet was resuspended in ice-cold [^{35}S]GTPγS binding assay buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4 °C for 10 min at 48,000 × *g*. The resultant pellet was resuspended in ice-cold [^{35}S]GTPγS binding assay buffer and stored at −70 °C until used. The membrane homogenate (3–8 μg protein/assay) was incubated at 25 °C for 2 h in 1 ml of assay buffer with 0.001–10 μM morphine, 30 μM guanosine-5′-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$ at 4 °C for 2 h. Filters were then washed three times with 5 ml of an ice-cold Tris–HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 h and the radioactivity in the samples was determined with a liquid scintillation analyzer. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments.

Total RNA in the whole brain was extracted using an SV Total RNA Isolation System (Promega, Madison, WI). To prepare first strand cDNA, 1 μg of RNA was incubated in 100 μl of buffer containing 10 mM DTT, 2.5 mM $MgCl_2$, dNTP mix, 200 units of reverse transcriptase II (Gibco-BRL, Grand Island, NY) and 0.1 mM oligo (dT)12-18 (Gibco-BRL). The μ-opioid receptor was amplified in a 50 μl PCR solution containing 0.8 mM $MgCl_2$, dNTP mix and DNA polymerase with synthesized primers: a sense primer of μ-opioid receptor, which is at position 299–320 (5′-AGACTGCCACCAACATCTACAT-3′) of the receptor, and an antisense primer at position 623–643 (5′-TGGACCCCTGCCTGTATTTTG-3′). Samples were heated to 94 °C for 2 min, 55 °C for 2 min and 72 °C for 3 min, and cycled 35 times through 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min. The final incubation was 72 °C for 7 min. The mixture was run on 1% agarose gel electrophoresis with the indicated markers and primers of the internal standard GAPDH. Three independent experiments were performed in this study. Semi-quantitation of the intensity of the bands for μ-opioid receptor/GAPDH was conducted by using a NIH image.

Data represent the mean counts with SEM. Statistical analyses were performed using two-way ANOVA with Bonferroni/Dunnett's test.

In the previous study, we found that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced pharmacological actions [19]. Here, we investigated the influence of prenatal and neonatal exposure to BPA in the several morphine-induced pharmacological actions. In the present study, morphine produced a dose-dependent place preference in control B0 mice (Fig. 1A), as reported previously [10,17]. Morphine at the low dosage that elicited neither place preference nor place aversion in control B0 mice produced a significant place preference in BPA-treated mice (Fig. 1A). As well as the rewarding effect, a significant increase in the morphine-induced hyperlocomotion was observed by chronic BPA treatment ($F_{1,340} = 6.617$, $P < 0.05$ vs. B0 group, Fig. 1B).

Recently, several investigations have provided evidence that the treatment of adult animals with BPA could not affect the reproductive function and social behaviors [2,3]. We have already confirmed that acute administration of BPA with adult mice could not affect the dopamine-related behaviors (data not shown). These findings indicate that prenatal and neonatal exposure to BPA may cause the neuronal toxicity specifically in the developmental process.

Many studies have pointed to the mesolimbic dopaminergic system, which originates from the VTA projecting mainly to the nucleus accumbens, as a critical site for the initiation of opioid addiction [7,13,14,16,20]. Morphine has been shown to indirectly activate dopamine neurons in the VTA as a consequence of inhibiting non-dopaminergic neurons, presumably γ-aminobutyric acid-containing neur-

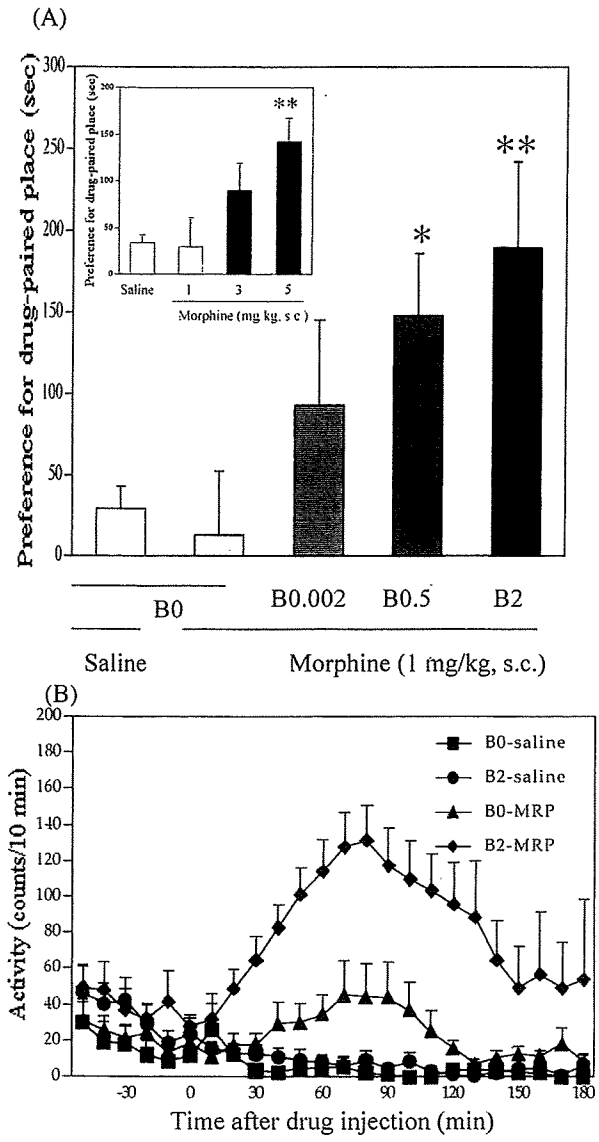

Fig. 1. (A) Effect of prenatal and neonatal exposure to BPA on the rewarding effect of morphine in mice. (Inner) Dose-response for the morphine-induced place preference in control B0 mice (*$P < 0.05$ vs. saline-treated mice). (Outer) Effect of BPA (0.002–2 mg/g of food: B0.002–B2) on the morphine (1 mg/kg, s.c.)-induced place preference. The BPA-non-treated group (hatched bar) did not show any place preference or place aversion with morphine at 1 mg/kg. The BPA-treated group (filled bar) showed a significant place preference induced by morphine at this dosage (*$P < 0.05$, **$P < 0.01$ vs. BPA-non-treated group). Each column represents the mean place preference score with SEM of six to ten mice. (B) Effect of BPA on morphine (10 mg/kg, s.c.)-induced hyperlocomotion. The BPA (2 mg/g of food)-treated group (diamond: B2 mice) revealed a significant potentiation of the morphine-induced locomotor-enhancing effect ($F_{1,340} = 6.617$, $P < 0.05$ vs. B0 group: triangle). Each point represents the mean activity counts for 10 min with SEM of nine to ten mice.

ons, leading to the increased dopamine release in the nucleus accumbens [10]. In order to investigate the mechanism of the enhancement of morphine's effect, we demonstrated whether prenatal and neonatal exposure to BPA could directly affect the opioidergic system associated with the mesolimbic dopaminergic system. In the [^{35}S]GTPγS binding assay, prenatal and neonatal BPA exposure failed to enhance μ-opioid receptor-mediated G-protein activation by morphine in the lower midbrain (Fig. 2A). As well as G-protein activation, the expression of μ-opioid receptor mRNA was not changed by chronic BPA treatment, suggesting that μ-

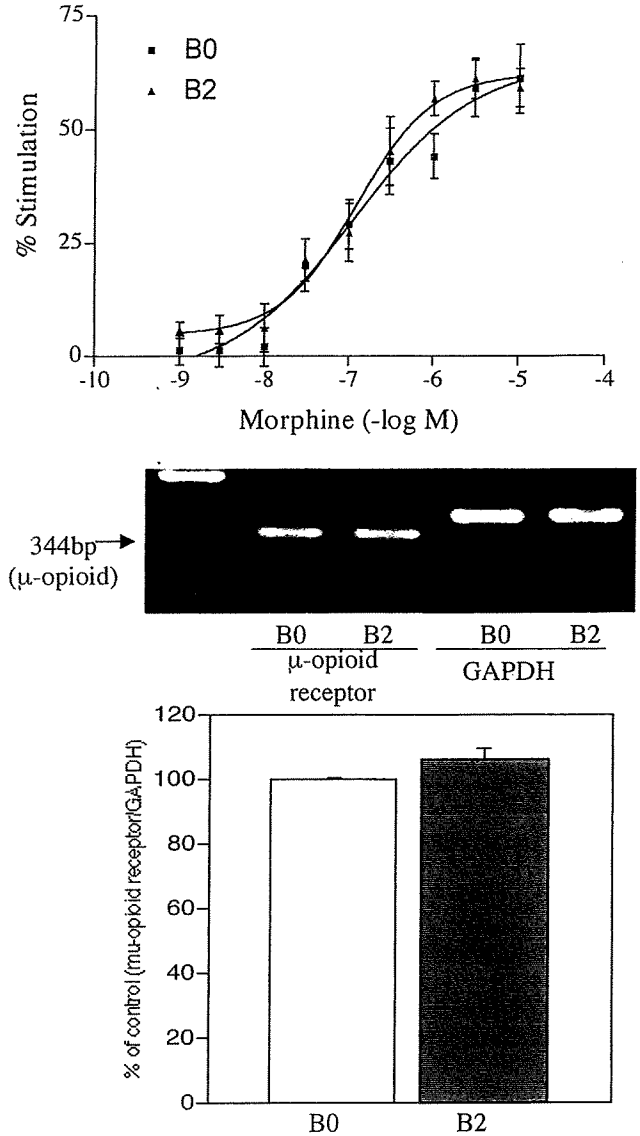

Fig. 2. No changes in the μ-opioid receptor in the mice prenatal and neonatal exposure to BPA. (A) Comparison of the stimulation of [^{35}S]GTPγS binding by morphine to membranes of the lower midbrain obtained from control (B0: square) and BPA-treated (B2: triangle) mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with morphine (10^{-9}–10^{-5} M). The values are expressed as % increase of the value in B0 mice. Data are expressed as the mean ± SEM of three independent samples. (B) Effect of prenatal and neonatal exposure to BPA on the expression of μ-opioid receptor mRNA in the mouse brain. (Upper) Representative RT-PCR for the μ-opioid receptor mRNAs in the whole brain minus cerebellum obtained from B0 and B2 mice. (Lower) There were no changes in the expression of μ-opioid receptor mRNAs in the whole brain obtained from B2 mice (filled bar) as compared to that from B0 mice (open bar). The values are expressed as a percentage of the value in B0 mice. Each column represents the mean ± SEM of three independent samples.

opioid receptor function is likely to be directly unaffected in this region. We have already reported that prenatal and neonatal exposure to BPA lead to an up-regulation of dopamine D_1 receptor in the limbic forebrain [19]. Taken together, these results indicate that the enhancement of the morphine-induced rewarding effect and hyperlocomotion may mainly result from the direct up-regulation of the dopaminergic transmission in the limbic forebrain.

BPA is considered to disrupt the endocrine systems via binding to estrogen receptor by mimicking estrogenic action. However, weak estrogenicity of BPA was confirmed with approximately 15,000 times less potency than 17β-estradiol [5]. Furthermore, BPA binds to estrogen receptor with low affinity and transactivates the estrogen responsive element-driven reporter gene in vitro [5]. Recently, it has been reported that BPA acts on thyroid receptor as an antagonist [9]. In addition, Song et al. have reported that BPA induces the expression and steroidogenesis of the orphan nuclear receptor Nur77 gene in mice Leydig cells [15]. Several subfamilies of Nur77, for example NGFI-B or Nurr1, have been shown to be highly expressed in the brain [21]. In particular, it has been accepted that NGFI-B is highly expressed in the basal ganglia, and is involved in the development of dopaminergic and opioidergic systems [1, 22]. These findings suggest that, although we cannot completely exclude the possibility of estrogenic action of BPA, the present action of BPA may mainly result from novel mechanisms associated with dopaminergic transmission.

In conclusion, the present data provide further evidence that prenatal and neonatal exposure to BPA can directly influence the development of the central dopaminergic system in the limbic area. These effects could lead to a change in sensitivity to morphine. Further investigation is necessary to fully understand the molecular mechanism of BPA in the CNS.

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Original Article

Effects of *in Utero* and Lactational Exposure to Bisphenol A on Thyroid Status 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 alters thyroid status in rat F_1 offspring. Dams were orally administered various doses of BPA (0, 4 or 40 mg/kg body weight per day) from gestation day (GD) 6 through postnatal day (PND) 20. The BPA and control groups did not differ significantly with respect to plasma thyroxine (T_4) concentration. The thyroid glands from the BPA groups had normal T_4 responses to exogenous thyroid-stimulating hormone *in vivo*. These results suggest that *in utero* and lactational exposure (indirect exposure) to BPA (4–40 mg/kg/day, GD 6 - PND 20) does not affect thyroid functions in the F_1 generation of male and female rats.

Key words: Bisphenol A, Thyroid, Thyroxine, Thyroid-stimulating hormone, Offspring, Rat

Introduction

Bisphenol A (BPA) is very widely used in the manufacture of polycarbonate and epoxy resins, dental sealants and other chemically derived products. BPA released from lacquer coatings 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 the weak estrogenic action of BPA eluted from a polycarbonate bottle into medium during an autoclaving procedure. They showed that BPA increases the number of progesterone receptors and promotes proliferation of a cultured cell line that originated from a human breast cancer (MCF-7)[3]. It has been estimated that total daily intake is 0.48 μg/kg body weight (BW) per day for 60-kg adults[4].

Reproductive toxicity of BPA has been reported in mice and rats, and low-dose effects of BPA *in vivo* have been observed in mice. BPA increased prostate and preputial gland weight and decreased daily sperm production efficiency in male mice offspring prenatally exposed to BPA at 2 or 20 μg/kg/day from gestation day (GD) 11 through GD 17[5, 6]. Other investigators, however, failed to confirm such effects in mouse offspring using identical experimental designs[7, 8]. Cagen *et al.* reported normal reproductive development in Wistar rat offspring born from mothers supplied with BPA in drinking water at a concentration range of 0.01 to 10 ppm (equivalent to approximately 0.001–4.022 mg/kg/day) for 10 weeks, from the premating day (at 9 weeks old) to the weaning day[9]. In addition, multigeneration reproductive toxicity studies of BPA have been conducted in rats[10, 11]. Both of these studies confirmed that there were no BPA-related effects on developmental and reproductive parameters at low doses of exposure.

Although the reported effects of BPA are not entirely consistent, it has received a great deal of attention with regard to its possible effects on reproductive glands and accessory genital glands, which are due primarily to its estrogenic activity. It has been known that BPA exerts weak estrogenic activity *in vivo*[12] and *in vitro*[13]. BPA may also disrupt thyroid homeostasis because of its structural similarity to thyroid

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Table 1. Treatment design and number of subjects examined

Measurement	Group	Dose (mg/kg/day)	No. of offspring tested			
			Age (wks)	1	3	9
Plasma T_4	Control	0	Male	6	5	5
			Female	8	5	5
	BPA	4	Male	9	6	3
			Female	8	6	5
	BPA	40	Male	1	5	5
			Female	9	5	5
TSH stimulation test	Control	0	Male	–[a]	–	4
			Female	–	–	7
	BPA	4	Male	–	–	2
			Female	–	–	4
	BPA	40	Male	–	–	4
			Female	–	–	4

[a]Not examined.

hormone. *In vitro* studies have demonstrated that BPA binds weakly to the thyroid hormone receptor and suppresses transcriptional activity that is stimulated by tri-iodothyronine (T_3)[14]. On the other hand, BPA does not inhibit the binding of T_3 to the thyroid hormone receptor and does not inhibit the hormonal activity of T_3 to induce growth and growth hormone (GH) production in the rat pituitary cell line GH3[15]. Although BPA is suspected to mimic thyroid hormone by modulating the thyroid hormone receptor, the *in vitro* effect of BPA on thyroid function/action is controversial, and the *in vivo* effect of BPA on thyroid status is unknown.

In the present study, we administered relatively high oral doses of BPA to pregnant rats and measured the effects on thyroid parameters including plasma thyroxine (T_4) levels at 1, 3 and 9 weeks of age in male and female offspring. To better assess thyroid function, we also measured *in vivo* T_4 responsiveness of thyroid glands to exogenous thyroid-stimulating hormone (TSH) at 9 weeks of age.

Materials and Methods

Animals

BPA (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 24 pregnant female rats (Crj: CD (SD) IGS strain, 9 weeks of age) at GD 3 were purchased from Charles River Japan, Inc. (Tsukuba) and housed separately and maintained under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and a 12-h light-dark cycle (08:00–20:00) conditions throughout the study. The presence of a copulatory plug defined GD 0. A standard laboratory diet (CE-2, Clea Japan, Inc., Tokyo, Japan) and drinking water were available *ad libitum*. Dams were randomly divided into four groups (6 pregnant rats per group) 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 per day of BPA in corn oil vehicle (10 ml/kg BW) once daily between 08:30 and 09:30 from GD 6 through PND 20, and the control group was given the same amount of corn oil during the same period. The litter size was standardized to ten (male:female = 5:5, if possible) between 10:00 and 11:00 on PND 7 (1 week of age). Examinations were performed as soon as possible after offspring were culled. On PND 21, the remaining offspring were weaned, and thereafter males and females were housed separately per litter. A pair of male and female offspring from each dam was dissected at 3 and 9 weeks of age. The remaining offspring were used for brain function and behavioral effects (not reported here). Table 1 shows the treatment design and number of subjects examined. The 400 mg/kg/day group was excluded from further analysis because of its excessive maternal toxicity[16].

Hormone assay

Plasma samples were prepared as described previously[16]. Plasma T_4 levels were determined by a chemiluminescence immunoassay (ACS; Centaur; Chiron Corp., Emeryville, CA). The plasma T_4 levels were also used as basal values for the TSH stimulation test (see below).

TSH stimulation test

The BPA-exposed and control groups of both sexes at 9

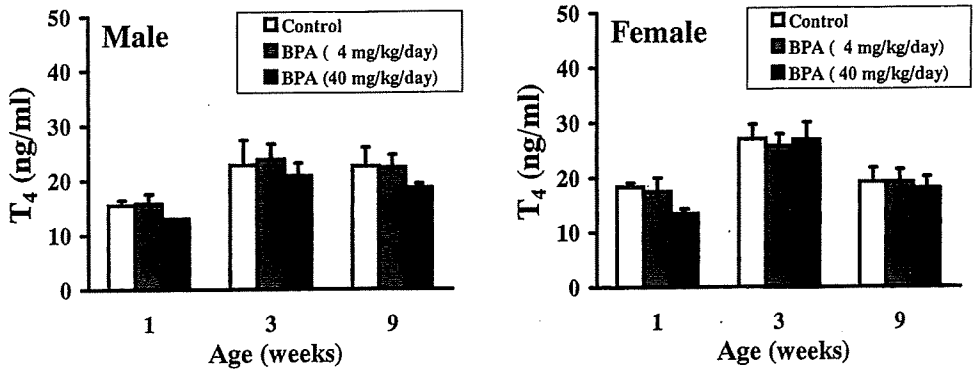

Fig. 1. Effects of maternal exposure to BPA on plasma T_4 levels in male and female offspring.
Each column and vertical bar represents the mean and SEM, respectively. There were no significant differences among groups.

weeks of age were injected with bovine TSH (bTSH; Sigma-Aldrich Corp., St. Louis, MO) intraperitoneally at 25.0 mIU/5 μl/g BW and intramuscularly at 12.5 mIU/5 μl/g BW. Blood samples were collected from the postcaval vein under euthanasia by ether inhalation at 6 h after bTSH administration and then stored at –20°C until the analysis. Plasma T_4 levels were measured as described above and are presented as the percentage of corresponding basal values.

Statistical analysis

For plasma T_4 determinations, the differences from the corresponding control group were statistically analyzed by analysis of variance followed by Dunnett's test (significance at $p<0.05$). In the TSH stimulation tests, the difference from the corresponding basal value was statistically analyzed using the Student's or Welch's *t*-test (significance at $p<0.05$).

Results

In male and female offspring, no statistically significant differences in plasma T_4 levels were observed between the control and BPA groups at 1, 3 and 9 weeks of age (Fig. 1). Plasma T_4 levels after TSH administration were significantly elevated compared with the corresponding basal values in the control and the BPA groups. No treatment-related differences were measured in the T_4 levels or in the relative T_4 increase in response to exogenous TSH in all groups of both sexes (Figs. 2, 3). In male offspring, plasma T_4 levels in the control, 4 and 40 mg/kg/day groups increased by 243%, 206% and 330%, respectively, compared with the corresponding basal values. Likewise, in female offspring, plasma T_4 levels increased by 285%, 311% and 275%, respectively (Fig. 3).

Discussion

Reproductive and developmental toxicity studies have been conducted using higher doses of BPA. Although maternal toxicity (reduction in maternal weight gain during gestation) was noted in rats exposed to BPA by gastric intubation at 160, 320 and 640 mg/kg/day from GD 6 through GD 15, there were no toxic effects on the development of their pups[17]. Kwon *et al.* showed that exposure at 320 mg/kg/day from GD 11 through PND 20 resulted in no apparent change in male and female pubertal development and reproductive function in SD rats[18]. We previously reported that exposure at 4 or 40 mg/kg/day from GD 6 through PND 20 did not cause changes in somatic growth or anogenital distance in rat offspring of both sexes[16], but these doses did affect testosterone homeostasis in the male testis[19]. Thus, much of the interest in BPA toxicity has focused on its putative effects on reproductive organs and genital glands. In the present study, we investigated whether *in utero* and lactational exposure to BPA affects endocrine status in the thyroid gland of rat offspring. Plasma T_4 levels were unaffected in the BPA groups (Fig. 1). A TSH stimulation test was then performed to examine thyroid function. Plasma T_4 levels were significantly elevated after exogenous TSH administration in the BPA-exposed groups, similar to the elevation seen in the control group (Fig. 2), suggesting that BPA exposure does not affect the synthesis and release of thyroid hormone in offspring *in vivo*. To our knowledge, this study represents the first attempt to better examine the effects of BPA on thyroid function *in vivo* in rat offspring exposed to relatively high levels of BPA.

Thyroid hormones play important roles in normal growth, neuronal development and metabolism in animals. During

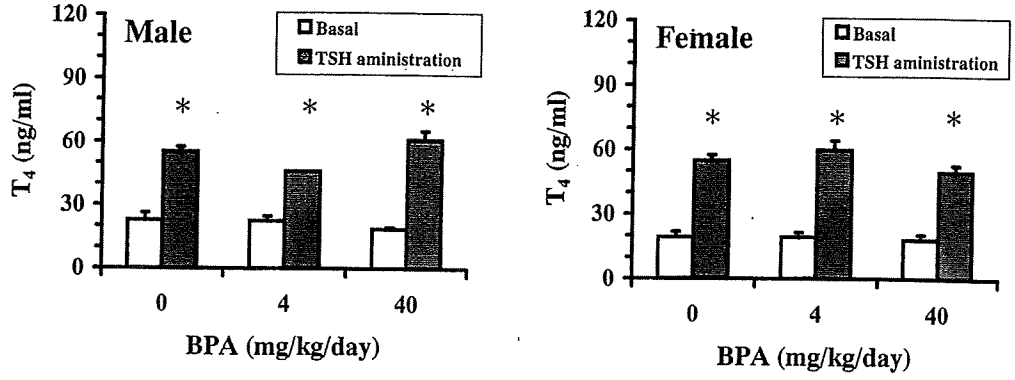

Fig. 2. Effects of maternal exposure to BPA on T_4 response to TSH in male and female offspring at 9 weeks of age.
Each column and vertical bar represents the mean and SEM, respectively. *Significantly different from the corresponding basal value ($p<0.05$).

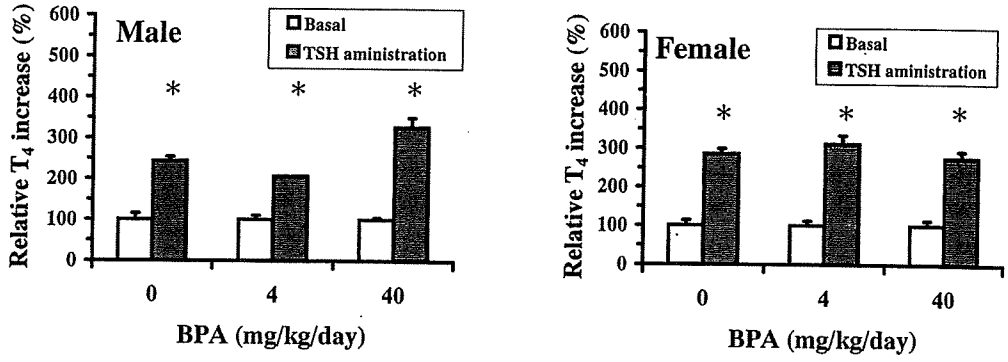

Fig. 3. Effects of maternal exposure to BPA on relative T_4 increase in response to TSH in male and female offspring at 9 weeks of age.
Each column and vertical bar represents the mean and SEM, respectively. *Significantly different from the corresponding basal value ($p<0.05$).

fetal and early neonatal periods, impaired thyroid hormone function may affect somatic growth[20]. BPA may act as an agonist or antagonist of the thyroid hormone receptor because of its structural similarity to thyroid hormone. Hence, given that thyroid hormone receptors are expressed ubiquitously and abundantly in various organs, BPA may perturb thyroid hormone action throughout the body tissue. Furthermore, BPA was observed to distribute rapidly in fetuses via placental transfer after a single BPA administration to pregnant female rats[21], mice and monkeys[22]; i.e., the placental barrier cannot block BPA transfer. Despite this high transplacental passage, there is sufficient glucuronyl transferase activity to metabolize BPA to BPA-monoglucuronide even in neonatal rats[23]. Although BPA may have the potential to disrupt thyroid hormone action through the thyroid hormone receptor, the results of this study confirmed that thyroid status was unaffected after *in utero* and lactational exposures of the offspring to BPA. The fact that normal somatic growth is observed in rat offspring following exposure of dams to BPA (even at high doses) [16] has led to the conclusion that the thyroid remains intact in the offspring.

In vitro studies have demonstrated the binding of BPA to the thyroid hormone receptor. Kitamura *et al.* reported that BPA does not inhibit the binding of T_3 to the thyroid hormone receptor and does not inhibit the hormonal activity of T_3 to induce growth and GH production of the rat pituitary cell line GH3[15]. On the other hand, Moriyama *et al.* used a competitive binding assay to confirm that BPA binds weakly to thyroid hormone receptors in rat liver nuclear extract[14]. Furthermore, BPA was shown to suppress T_3-stimulated transcriptional activity in transient expression assays[14]. The discrepancies between these *in vitro* studies indicate that further investigation is required to clarify the possible mechanism(s) of BPA action on the thyroid hormone receptor. Our results here are in accordance with an *in vitro* study reported by Kitamura *et al.* [15], however, we have no plausible

explanation for the differences between these *in vitro* studies and the *in vivo* outcome that we describe here.

In conclusion, the results of the present study suggest that *in utero* and lactational exposure to BPA does not have an effect on thyroid status in the F_1 generation of male and female rats under our experimental conditions.

Acknowledgments

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Original Article

Comparative Investigation of Several Sperm Analysis Methods for Evaluation of Spermatotoxicity of Industrial Chemical: 2-Bromopropane as an Example

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Abstract: Reproductive toxicity of 2-bromopropane (2BP), a substitute for ozone layer-depleting chloro-fluorocarbon, was found among the workers in an electronics factory in Korea in 1995. Furthermore the importance of testicular toxicity has been realized since the problem of endocrine disruptors arose all over the world, but manual methods must rely on subjective assessment. Recently, computer-assisted sperm analysis (CASA) was proposed but this system requires vast investment. We then investigated the applicability of the MTT method with a microplate and sperm quality analyzer (SQA) as simple, rapid, and economic instrumental methods for the examination of sperm quality in rats, comparing it with the manual microscopic method and CASA. Epididymal fluid derived from male F344/N Slc (Fischer) rats intraperitoneally injected with 2BP in the dose range of 125–1,000 mg/kg/d twice a week (total 8 times) were examined by these methods as a model experiment. Sperm count measured by the manual method and CASA in the epididymal fluid, absorbance by the MTT method and sperm motility index value by the SQA method were significantly lower in the 2BP 1,000 mg/kg administered group than in the control group. This result suggests that the MTT method can detect oligospermia. With the microplate and microplate reader, the efficiency of detection becomes much better. Sperm analyses by the MTT method with the microplate reader and the SQA method are available for reproductive toxicity study in rats.

Key words: 2-Bromopropane, Tetrazolium salt, MTT (3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl tetrazolium bromide), SQA (sperm quality analyzer), CASA (computer-assisted sperm analysis), Manual microscopic method, Reproductive toxicity, Rat

Introduction

Certain substances found in the environment can upset normal endocrine balance and become a health hazard. An example of growing concern is their effect on sperm[1]. Some workers in semiconductor factories in Korea were found to have affected in their reproductive functions after exposure to 2-bromopropane (2BP)[2,3]. Subsequently, the reproductive effects of 2BP were confirmed to the animal experimental studies[4–7]. These reports prompted close reappraisal of the efficacy and feasibility of mass screening for toxicity to male reproductive functions in industrial populations.

Surveying the method of investigating sperm activity, each method now in use has its own serious shortcomings. For instance, the conventional, manual method of sperm count and assessment of motility under the optical microscope is fraught with inevitable subjective variations which would make inter-institutional comparison of data practically

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impossible. The introduction of computer-assisted sperm analysis (CASA) eliminated variations due to subjective evaluation but this apparatus is expensive[8–13] and has not enjoyed wide acceptance. Meanwhile, the sperm quality analyzer (SQA), which measures sperm count and motility by the optical method, was introduced as a simple and inexpensive alternative[14, 15]. The third approach is the biochemical method (MTT method) which measures color changes in the tetrazolium reaction to mitochondrial reductase by absorption spectrometry reflecting the overall numerical and functional power of sperm activity[16, 17]. Although these methods of sperm testing have proliferated, their performance and efficacy have been evaluated individually and never collectively using the same test samples. In particular, comparative investigation of the MTT method with CASA has never been performed. Their performance in terms of mass handling of large numbers of samples has not been evaluated properly.

We attempted to develop a method for measuring toxicity to the sperm by combining an absorption spectrophotometer with a microplate reader so that a large number of specimen can be processed rapidly. The method is objective, simple, inexpensive and efficient and can be applied to mass screening of workers in suspicious environments. In the days when more and more clinical tests for male reproductive disability need to be performed on an everyday basis, the ability to processing a large number of samples will be an important prerequisite in the selection of test methods. Furthermore, the MTT method, with its speed and simplicity to deal with a large number of facilities, is a technique suitable for the animal studies of male reproductive disturbance induced by various chemicals. In this study, we induced reproductive toxicity with 2BP as a representative of bromopropanes, which are used widely in the industrial workplace.

We report the results of a study carried out on rats given repeated doses of 2BP using the MTT method with a microplate reader in comparison with other methods of sperm testing including CASA. The advantages and merits of various methods were compared and problems in performing the tests will be discussed.

Materials and Methods

Chemicals and supplements

2BP and MTT were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Dojin (Kumamoto, Japan), respectively. Olive oil, HCl and isopropyl alcohol were from Wako Pure Chemical (Osaka, Japan). Bovine serum albumin Fraction V (BSA), Medium 199 and phosphate buffered saline (PBS) were from Seikagaku Kogyo (Tokyo, Japan), GIBCO (Grand Island, USA) and Nissui Pharmaceutical (Tokyo, Japan), respectively.

Instruments

The semen analyzer (HTM-IVOS Ver. 10.9i) was from Hamilton Thorne Research (Beverly, MA, USA). SQA was the product of Medical Electronic Systems (Migdal Haemek, Israel). The microplate reader (Immunoreader NJ2000) was purchased from Nalge Nunc (Tokyo, Japan). The optical microscope (Eclipse E600) was from Nikon (Tokyo, Japan).

Experimental protocol

F344/N Slc (Fischer) male rats (11 wk of age) from Japan SLC (Shizuoka, Japan) were kept in cages under standard conditions and received pellets (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. The body weight was monitored just before each administration and sacrifice. Each of 4–5 rats (12 wk of age)/group received intraperitoneal instillations of 2BP dissolved in olive oil twice a week for 24 d in doses of 125, 250, 500 and 1,000 mg/kg. Control rats received an equal volume of olive oil. So each rat received a total of 8 injections. After a one week rest period following the last dose, the animals were sacrificed under ether anesthesia and the testes, epididymis and epididymal cauda were separated and weighed immediately. And then relative organ weights were calculated.

Preparation of epididymal cauda sample

Epididymal cauda was minced with scissors to release sperm in 2 ml of Medium 199 containing 0.5% BSA at 37°C. This sperm suspension sample served for the MTT and SQA methods. The aliquot of this sample was stored at –80°C. Before sperm count analysis, this aliquot was diluted 1:4 with PBS, further homogenized at room temperature, and served as a sample for the manual method and CASA.

Manual method

After staining with trypan blue the specimen was spread on a hemocytometer and the sperm heads were counted manually under the optical microscope. The data were expressed as the total number of sperm per one cauda epididymal tissue.

CASA

Each sample was stained with the attached staining kit (Supra Vital IDENT Stain Kit, Hamilton-Thorne Research, Bevely, MA, USA), dropped into a disposable counting chamber CELL-VU (Millennium Sciences Corp. NY, USA) and

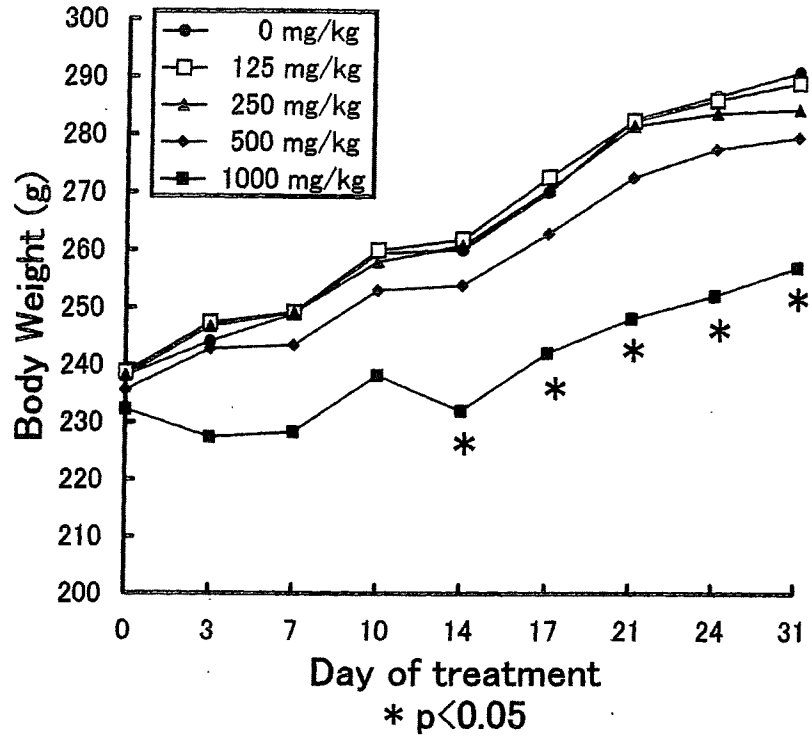

Fig. 1. Mean body weight of F344 rats exposed to 2BP ip.

mounted on the Semen Analyzer. Sperm heads fluorescence under an ultra-violet beam were counted in RAT-IDENT mode automatically. The data were expressed as the total number of sperm per cauda epididymal tissue sample.

SQA method

The disposable SQA capillary (Medical Electronic Systems, Migdal Haemek, Israel) containing the specimen was inserted into the slot in the SQA and the sperm motility index (SMI) was determined.

MTT method

Fifty microliters of the sperm suspension sample in the sterile 96-microplate well was incubated with MTT reagent (5 mg/ml in PBS, 25 micro liter) at 37°C for two hours. Then the reaction was stopped by rapid cooling. After the addition of 0.04 M HCl-isopropylalcohol and pipeting exhaustively to dissolve the formazan thus formed in the process, the absorbance in each well was estimated at 574 nm by the microplate reader.

Correlation among data from various methods

In an attempt at evaluation of various methods, we carried out the following experiments. Epididymis cauda obtained from an untreated rat (17 wk of age) was dissected with a pair of scissors in 2 ml of medium to suspend sperm. The sample consisted of this undiluted suspension and its dilutions with Medium 199 containing 0.5% BSA to 4 strength. Data on sperm count, SMI and absorbance obtained by pair of investigating methods were compared and correlation was sought.

Statistical analysis

The data were analyzed by one-way ANOVA. The statistical significance of difference between the control and 2BP-treated groups was determined with Fisher's PLSD test. In all cases, $P < 0.05$ was considered statistically significant.

Results

Body and organ weights

Body weight decreased in the groups with a dose of 1,000 mg/kg (Fig. 1) on and after day 14 as compared to the control group. The relative weights of testis (TE, right (R) and left (L)), epididymis (EP, right (R) and left (L)), and epididymis cauda (EPC, right (R) and left (L)) are shown in Table 1. The weight of both the right and left testis decreased in the 500 and 1,000 mg/kg dose groups. In the 250 mg/kg dose group, only the left testis weight decreased significantly, but no significant difference was found in the right testis.

Table 1. Relative weight (%) of reproductive organ in 2BP-treated (mg/kg) rats

2BP (mg/kg)	TER	TEL	EPR	EPL	EPCR	EPCL
0	0.503 ± 0.010	0.521 ± 0.018	0.172 ± 0.007	0.176 ± 0.011	0.080 ± 0.004	0.076 ± 0.005
125	0.505 ± 0.020	0.512 ± 0.020	0.186 ± 0.012	0.176 ± 0.017	0.089 ± 0.003*	0.082 ± 0.006
250	0.493 ± 0.023	0.480 ± 0.025*	0.183 ± 0.011	0.184 ± 0.014	0.087 ± 0.006	0.089 ± 0.011*
500	0.407 ± 0.021*	0.409 ± 0.038*	0.168 ± 0.012	0.179 ± 0.008	0.086 ± 0.006	0.076 ± 0.003
1000	0.193 ± 0.010*	0.199 ± 0.019*	0.131 ± 0.009*	0.130 ± 0.007*	0.056 ± 0.006*	0.055 ± 0.004*

TER: Right Testis, TEL: Left Testis, EPR: Right Epidydymis, EPL: Left Epididymis. EPCR: Right Epididymal Cauda, EPCL: Left Epididymal Cauda. Each value represents the mean ± SD. *: Significantly different at $p<0.05$.

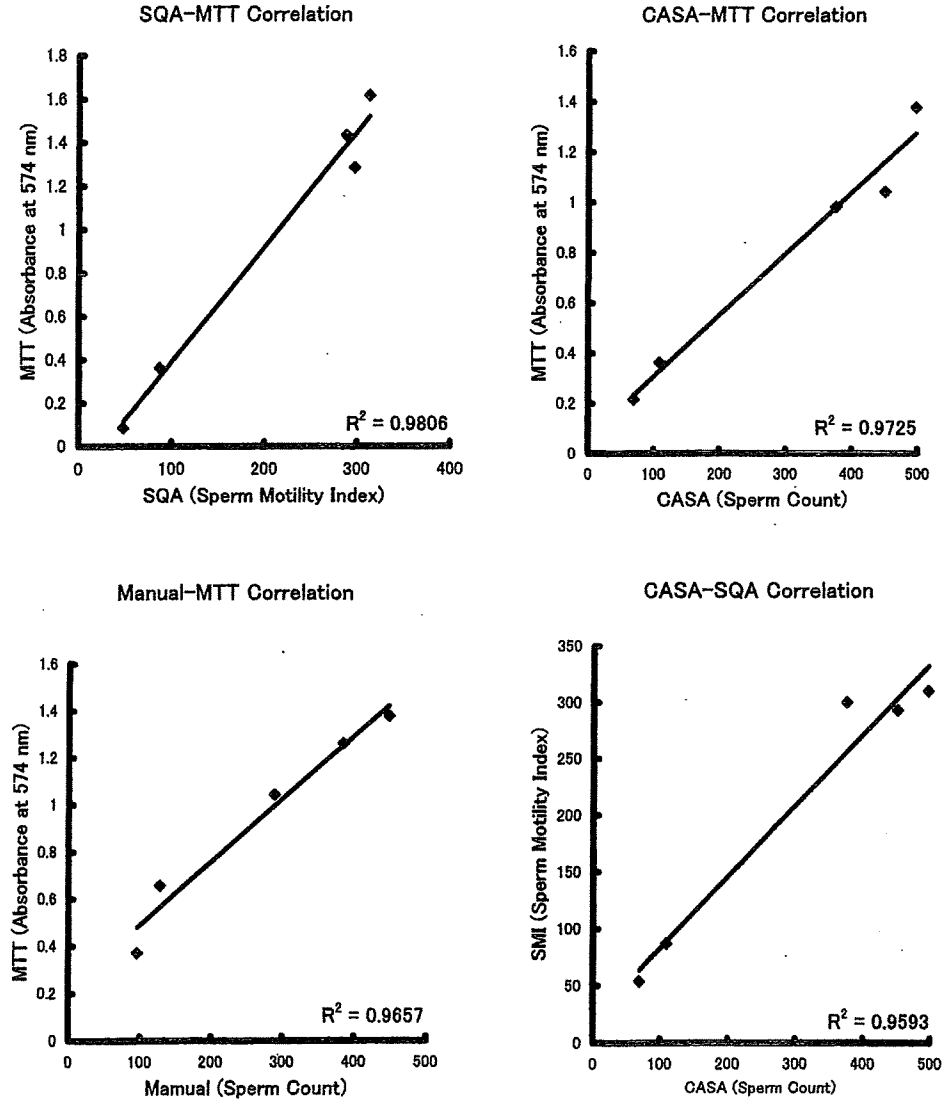

Fig. 2. Relationship between parameters of several methods.

And the weights of the epididymis and epididymal cauda decreased in the 1,000 mg/kg group, but some reverse results were found in lower dose groups (EPCR: 125 mg/kg, EPCL: 250 mg/kg), but they were not in both sides of the tissues.

Correlation between the sperm analysis methods

Figure 2 shows the correlation between the two methods. A high correlation was found between the SMI value found by the SQA method and absorption by the MTT method

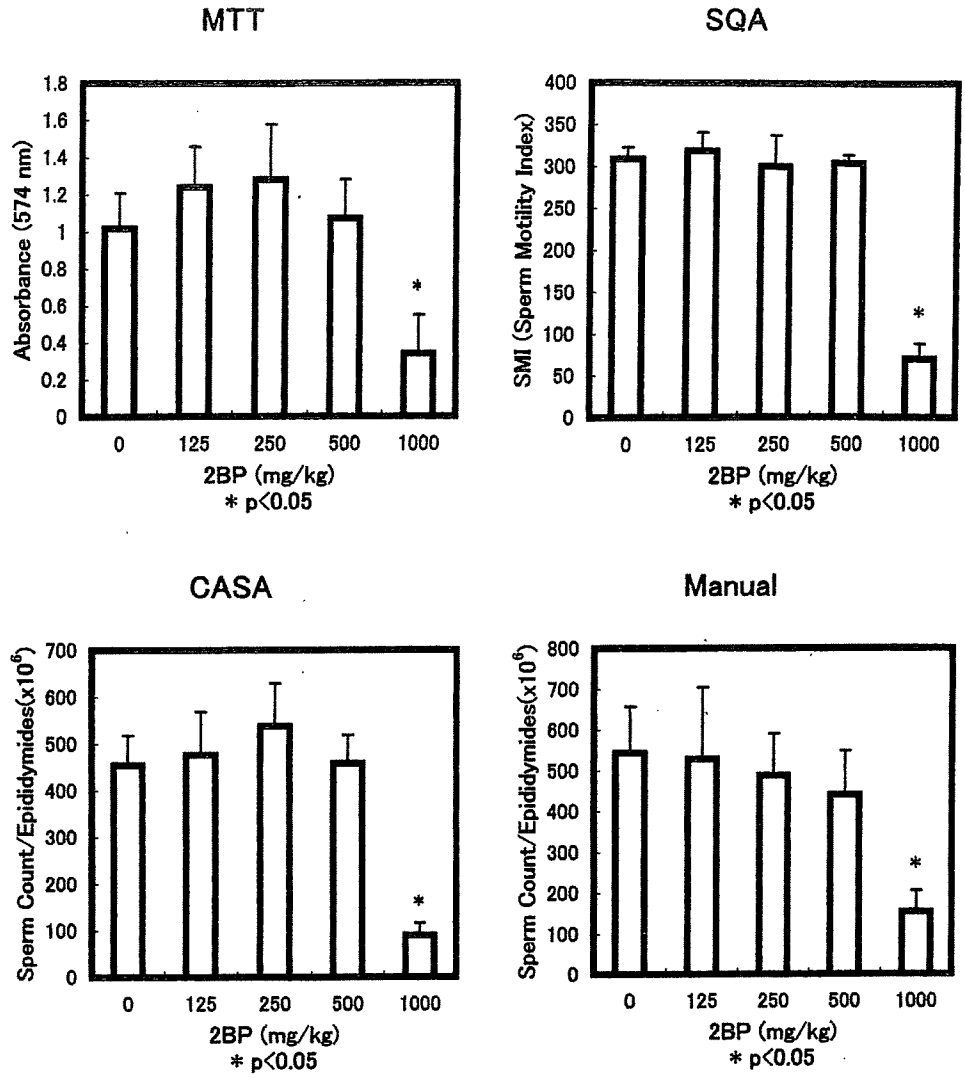

Fig. 3. Effect on rat sperm of 2BP (Comparison of results obtained by the MTT, SQA, CASA and Manual methods).

(R^2=0.9806). Similarly, MTT absorbance correlates well with the Sperm count by CASA (R^2=0.9725) and the manual method (R^2=0.9657). All the methods had common linearity mutually.

Detection of sperm toxicity of 2BP by several methods

As shown in Fig. 3, sperm count by the manual method, sperm count by CASA, SMI by the SQA method and absorbance by the MTT method were similarly low only in the 1,000 mg/kg group as compared with the control group. There was no significant reduction in the parameters in the groups given 2BP at the dose of less than 500 mg/kg.

Discussion

Only the testes weight could indicate sperm toxicity in the 500 mg/kg dose group, whereas while other methods failed to detect the change (Table 1 and Fig. 3). Although the weight of testes was the most sensitive parameter indicating the sperm toxicity, this requires autopsy and therefore has no clinical usefulness. A non-invasive method such as sperm count under an optical microscope is more practical and is widely accepted, but inherent shortcomings of this method are obvious: counts are susceptible to subjective variations and their inter-institutional or inter-observer comparisons and analyses are unreliable if ever possible, and it is not suitable for processing a large number of specimens in mass screening.

SQA is a simple and practical method, but it is no match in efficiency for the MTT method. In comparison, the MTT method excels SQA on account of rapidity and simplicity and therefore its ability to handle a large number of samples simultaneously. CASA gives not only the sperm count, but other information such as the motility rate and even morphological indexes[8–13]. Unfortunately this requires rather expensive equipment and has not achieved wide acceptance. MTT, one of the tetrazolium salts, is known to form formazan and is turned blue in the somatic cells by mitochondrial reductase[16]. The same reaction is observed in a suspension of sperm and the extent of coloring reflects the number and viability of the sperm[17]. The MTT method is utilized in many toxicity studies on somatic cells, but its use in sperm cell studies has been reported in only one paper. We introduced the use of the microplate and established a distinctly more efficient measurement system. If only qualitative analysis is required, direct observation may suffice, dispensing with absorption spectrometry. Possibly other tetrazolium salts may be found equally or more useful as the substrate and may replace MTT. We proposed the use of the MTT method for sperm analysis and established the protocol with microplates to facilitate processing a large number of samples rapidly as is required in mass screening. In the sperm count we obtained high correlation between the results by the MTT method and those by SQA, CASA, and the manual method as shown in Fig 2. We believe that the MTT method can replace these other methods where only the sperm count is required, but the MTT reaction is dependent on the activity of mitochondrial reductase in the sperm. This method cannot be expected to distinguish those sperm with abnormal morphology or diminished motility from normal, healthy sperm, as long as they have metabolic activity. On the other hand, the SMI value which is obtained by SQA has a positive correlation with both sperm count and sperm motility and is recognized as a strong predictor of fertility of the semen. The manual method can distinguish sperm deformity as well as give the sperm count. The advantage of the MTT method, on the other hand, is the efficiency in processing a large number of specimens and therefore may be a powerful tool for preliminary screening. The results of our animal experiments also established that the MTT method could detect sperm toxicity caused by introduced chemical agents to an extent comparable to other methods such as the manual method or CASA.

In conclusion we assert that the MTT method using the microplate reader provide a new tool in detecting sperm toxicity with sensitivity comparable to conventional or more expensive methods and is especially suitable for workplace mass screening. But manual dissolution of formazan is an extra step required in the MTT method. It is not readily amenable to automation. And this is a cause of errors in measurement. Recently tetrazolium salts which produce water-soluble formazan have been developed and their usefulness in toxicity tests on somatic cells has been reported. When these newer salts are used the process of dissolution of formazan is not necessary. Enhanced simplicity and improved accuracy of the method are expected. We plan to continue further studies on sperm toxicity using various tetrazolium salts to replace MTT.

In our experiments, distinct sperm toxicity was observed only in the group of rats given a large dose (1,000 mg/kg) of 2BP whereas a significant reduction in the weight of testes had already been found in the 500 mg/kg group (Table 1). This suggests that 2BP exerts its effect in the spermatogenesis stage. And reverse effects were observed in several cases in organ weight (EPCR: 125 mg/kg, EPCL: 250 mg/kg). This phenomenon is thought to result in a transitory effect.

Furthermore, we have started the study of reproductive toxicity induced by bromopropanes other than 2BP and have found that the MTT method is equally applicable to in these studies. We plan to present additional data on 2BP and other chemicals and further discussion on the mechanism of reproductive toxicity in our ensuing reports.

Acknowledgement

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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