TABLE VI. Summary of Biochemical and Hematological Indices in Male Workers (n=13), Yixing City, Jiangsu Province, China, 1999

	Unit	Mean ± SD	Minimum value	Maximum value	Percentage below reference value	Percentage above reference value	Reference value
ALT	IU/L	25.4 ± 4.1	20	35	0	0	10–40
γ-GTP	IU/L	30.5 ± 24	9	86		7.7	≤70
ALP	IU/L	179.5 ± 37.1	131	246	0	0	80–260
LDH	IU/L	303.9 ± 50.2	231	398	0	0	230–460
CK	IU/L	200.9 ± 144.4	75	547	0	23	57–197
CKM	ng/ml	188.0 ± 196.9	63.1	796.4			
TP	g/dl	8.3 ± 1.4	7.3	12.4	0	23	6.7–8.3
Albumin	g/dl	5.1 ±0.4	4.3	5.6	0	0	3.7–5.5
Total bilirubin	mg/dl	0.69 ± 0.36	0.1	1.4	0	7.7	0.2–1.0
BUN	mg/dl	14.3 ± 3.5	8.9	21.5	0	7.7	6–20
Creatinine	mg/dl	0.88 ± 0.08	0.8	1	0	0	0.6–1.3
Creatine	mg/dl	0.36 ± 0.18	0.22	0.9	46.2	0	0.31–1.10
Fe	μg/dl	102.7 ± 45.3	40	172	15.4	0	54–200
Ft	mg/dl	115.8 ± 50.2	24	210	7.7	0	27–320
TIBC	mg/dl	310 ± 33	247	355	7.7	0	253–365
LH	mIU/ml	6.1 ±2.3	3.5	10	0	53.8	1.8–5.2
FSH	mIU/ml	8.1 ±6.9	3.2	28	0	30.8	2.9–8.2
Testosterone	pg/ml	90.6 ± 114.4	242	604	15.4	0	250–1100
WBC	$10^3/\mu l$	391.6 ± 113.8	3.1	10.2	7.7	7.7	3.9–9.8
RBC	$10^4/\mu l$	6.06 ± 2.14	3.67	5.75	0	7.7	4.3–5.7
Hb	g/L	4.67 ± 0.6	114	163	30.7	0	135–176
Hct		142.2 ± 14.8	0.337	0.482	30.7	0	0.398–0.518
MCV	Fl	0.418 ± 0.042	83.3	98.5	0	0	82.7–101.6
MCH	Pg	89.9 ± 4.4	28.1	33.1	0	0	28.0–34.6
MCHC	%	30.6 ± 1.7	33.3	34.8	0	0	31.6–36.6
Plt	$10^3/\mu l$	34 ± 0.5	96	212	23.1	23.1	13.1–36.2

ALT, L-alanine 2-oxoglutarate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; LDH, lactose dehydrogenase; CK, creatine kinase; BUN, blood urea nitrogen; TIBC, total iron-binding capacity; LH, luteinizing hormone; FSH, follicle stimulating hormone; WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

adhesive solvent, headache was the most frequently reported symptom, but concentration-dependency was not clear in that study [Trout, 1999]. The present study, as well as NIOSH studies [Reh, 2001] and our case report [Ichihara et al., 2002], suggested that neurological- or mucosal irritation-related symptoms could be caused by 1-bromopropane exposure although their specificity is uncertain.

Sclar reported a case exposed to 1-bromopropane who complained of numbness, weakness of the lower extremities, dysphagia, and urinary difficulties [Sclar, 1999]. The patient developed a complex set of symptoms involving both the peripheral and the central nervous systems. The author initially treated the patient as a case of multiple sclerosis, but the final diagnosis was considered encephalo-myelo-radiculo-neuropathy. Other cases found in North Carolina had symptoms suggestive of disorders of peripheral nerves and/or the central nervous system [Ichihara et al., 2002]. All three patients complained of numbness, paresthesia/dysesthesia, difficulty in speaking, diarrhea, thirst, vertigo or dizziness, light headedness, feeling intoxicated, headache, memory loss, and insomnia. Among these, two with severe symptoms had urinary incontinence, dysphagia, nasal irritation, sore throat, strange taste, and hot face. In the present study, workers complained of mucosal irritation, dizziness, and headache, but did not complain of specific neurological signs such as numbness, paresthesia or dysesthesia, urinary or speech difficulties. This difference might be due to the maximum exposure levels, since North Carolina cases [Ichihara et al., 2002] developed symptoms when the exposure levels were very high because of the ventilation problem.

In the present study, nine female (no. 4, 5, 7, 8, 10, 13, 15, 17, 21) and four male (no. 27, 30, 33, 37) workers had mild anemia, which was defined as Hb or Ht levels below than the reference range, and three females had irregular menses or amenorrhea. While it was possible to explain anemia in five females (no. 4, 5, 7, 15, 17) and one male (no. 33) by iron

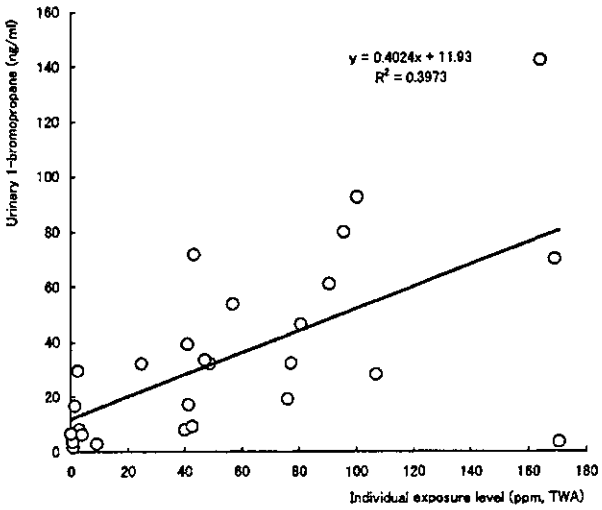

FIGURE 1. Relationship between urinary 1-bromopropane levels and 1-bromopropane exposure levels. There was a significant correlation between urinary 1-bromopropane concentrations and time-weighted average of 1-bromopropane trapped in the passive sampler tube. $P < 0.05$.

deficiency, its cause could not be determined in the other cases. In rats, exposure to 2-bromopropane, but not by 1-bromopropane at or less than 800 ppm, resulted in reduction of hematological indices [Ichihara et al., 1997; Nakajima et al., 1997]. Past exposure to 2-bromopropane is another possible explanation for anemia particularly in cases with longer service but without iron deficiency, such as one male repairman (no. 27). However, it was difficult to explain the anemia in one female (no. 21) and two male cases (no. 36, 37), because they had worked for less than 12 months in the present plant and they did not show iron deficiency or other specific causative agents. Instead, the female showed rather higher ferritin (Ft), which might be related to menopause similar to workers (1) and (2). Thus, it was difficult to uncover confounding factors other than iron when considering the association between length of service and anemia. We also faced similar difficulties in interpreting the correlation between duration of employment and Plt count or creatinine levels. Smoking could be a confounding factor, affecting Ht, but this effect could not be controlled. Failure to control confounding factors such as iron deficiency or smoking habit requires careful evaluation of the changes of hematological indices.

TABLE VII. Comparison of the Number of Female Operators With Various Complaints Between 1996 and 1999; Jiangsu Province, China

	1996[a]	1999
n	9	21
Strange smell	6	14
Irritated nose	6	10
Sore throat	3	9
Painful eyes	5	9
Dizziness or vertigo	5	6
Strange taste	1	5
Light headed	2	4
Heavy headed	3	3
Headache	5	3*
Dim eyesight	3	2
Flushed face	1	1
Feeling intoxicated	1	1

[a]Only female operators were included in this analysis.
*$P < 0.05$, Fisher's exact test for comparison of frequency between 1996 and 1999.

Comparison of data between 1996 and 1999 did not show any worsening of hematological indices by exposure to bromopropanes during the last 2.5 years. Only nine of the workers investigated in 1996 continued to work in the same factory in 1999, but healthy worker effects was not suggested by the lack of difference in hematological indices or symptom frequency between the workers who continued to work in the same factory and those who had left.

FSH was the only biochemical index that correlated with 1-bromopropane exposure levels, but no such correlation had been identified previously in animal studies.

The present study identified workers in a 1-bromopropane-producing factory who had anemia or amenorrhea, but could not confirm that these abnormalities were due to exposure to 1-bromopropane because of the small number of subjects and the lack of appropriate controls.

Our previous studies showed that rats exposed to 1-bromopropane have reduced CK activity in plasma and the central nervous system [Ichihara et al., 2000a; Wang et al., 2002, 2003]. Accordingly, we considered that plasma CK activity might be a good biological marker of 1-bromopropane in humans. However, in the present study, no significant correlation was present between CK activity or CK–M subunit and the exposure level or duration of employment. While CK activity could be a suitable biomarker of toxicity, evaluation of individual with longer and higher exposure levels and in appropriate control with matched confounding factors, is necessary to confirm this possibility. Our results also showed that the urinary excretion of 1-bromopropane correlated significantly with the exposure concentration measured on the same day, suggesting that urinary 1-bromopropane may be a good biomarker of acute exposure to 1-bromopropane.

Admittedly, there are limitations in this survey. Samples had to be transported at 4°C from China to Japan, a process that took about 2 weeks, to measure urinary 1-bromopropane concentrations or individual exposure levels. However, the stability tests showed that urinary 1-bromopropane levels diminished by only ~10% per week in vials kept at 4°C degree while the passive sampler did not show significant

TABLE VIII. Changes in Hematological Indices in Workers who Continued to Work in the Same Factory for More Than 3 Years; Jiangsu Province, China

No.	Sex	Job title	Age (years)	RBC ($\times 10^6/\mu l$)	HB (g/L)	Ht	WBC ($\times 10^3/\mu l$)
3	F	Operator	42	+0.27 (3.86)	−1 (131)	0.027 (0.378)	1.1 (7.1)
4	F	Operator	38	+0.14 (3.75^L)	+1 (112^L)	−0.006 (0.329^L)	+0.1 (5.2)
5	F	Operator	28	−0.33 (3.92)	−11 (120)	−0.026 (0.343)	+0.2 (4.8)
7	F	Operator	43	+0.07 (3.6^L)	−9 (115)	−0.9 (0.329^L)	−1.4 (5.9)
22	F	GC analyzer	27	+0.14 (3.94)	−1 (124)	+0.004 (0.357)	−4.1 (10.2^H)
23	F	Accountant	36	+0.03 (4.13)	0 (130)	+0.008 (0.373)	+1.8 (6.7)
25	M	Manager	38	+0.85 (4.9)	+15 (148)	+0.056 (0.426)	−1.5 (7.7)
26	M	Repairman	49	−0.08 (5.03)	−8 (161)	−0.025 (0.467)	+0.1 (6.1)
27	M	Repairman	48	+0.21 (3.9^L)	+1 (127^L)	+0.012 (0.358^L)	−1.3 (5.6)

Values were obtained by subtracting 1996 value from that of 1999. Numbers in parentheses depict the values in 1996. Superscript L and H indicate lower and higher than the reference values, respectively.

There were no significant differences between 1996 and 1999 values (paired *t*-test), both when the analysis was applied to all above nine workers and when the subjects were limited to the female operators and GC analyzer.

changes over the 2-week period. Thus, the present results might underestimate the level of urinary 1-bromopropane in relation to exposure levels.

In conclusion, the change from 2-bromopropane to 1-bromopropane production in the tested factory might reflect the recent preference for 1-bromopropane over 2-bromopropane as an alternative to ozone-depleting solvents. Irritation of the nose, throat and/or eyes and symptoms related to the central nervous system were detected in the factory workers, but no severe chronic symptoms of neurological damage, as reported previously in single case reports were detected in workers exposed to 1-bromoproane at $\leq$170 ppm. Urinary 1-bromopropane could well serve as a biomarker of individual exposure levels.

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Exposure to 1-Bromopropane Causes Ovarian Dysfunction in Rats

Tetsuya Yamada,*[,1] Gaku Ichihara,* Hailan Wang,* Xiaozhong Yu,*[,2] Kei-ichiro Maeda,† Hiroko Tsukamura,† Michihiro Kamijima,* Tamie Nakajima,* and Yasuhiro Takeuchi*[,3]

*Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan; and †Department of Biological Resources and Environmental Sciences, Nagoya University Graduate School of Bioagricultural Sciences, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

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Although 1-bromopropane has been used in chemical and electronic industries as an alternative to ozone layer-depleting solvents, its toxicity on female reproductive organs has not been fully elucidated. The aim of this experiment was to determine the effect of 1-bromopropane on female reproductive function in rats. Forty female Wistar rats were divided into four equal groups. Each group was exposed daily to 0, 200, 400, or 800 ppm of 1-bromopropane for eight h a day. After exposure for 7 weeks, all rats in the 800-ppm group became seriously ill and were sacrificed during the 8th week. The other dose groups were exposed for 12 weeks. In the 800-ppm group, but not in the other two exposed groups, body weight was significantly less than the control at each time point from 2 to7 weeks after the beginning of exposure. Tests of vaginal smears showed a significant increase in the number of irregular estrous cycles with extended diestrus in the 400- and 800-ppm groups. Histopathological examination of the ovary showed a significant dose-dependent reduction of the number of normal antral follicles and a decrease in the number of normal growing follicles in the 400-ppm group. No significant change was found in plasma concentrations of LH or FSH in any group when compared with the control. Our results indicate that 1-bromopropane can induce a dose-dependent ovarian dysfunction in nonpregnant female rats associated with disruption in follicular growth process.

Key Words: **1-bromopropane; chlorofluorocarbon alternative; reproductive toxicity; female; estrous cycle; ovary; ovarian follicle; inhalation exposure; rat.**

To preserve the ozone layer, certain chlorofluorocarbons and 1,1,1-trichloroethane have been banned from production in industrially developed countries since January 1996. Alternative compounds have been used as cleaning solvents in electronic industries. In Korea, amenorrhea, oligospermia, and anemia occurred in workers exposed to 2-bromopropane, which was used as an alternative to 1,1,1-trichloroethane (Kim *et al.*, 1996). Subsequent studies revealed that 2-bromopropane is very toxic to reproductive and hematopoietic organs (Ichihara *et al.*, 1997; Kamijima *et al.*, 1997; Nakajima *et al.*, 1997; Omura *et al.*, 1997; Son *et al.*, 1999; Takeuchi *et al.*, 1997; Yu *et al.*, 1999). Accordingly, the use of 2-bromopropane as a solvent was strictly limited in industry. Instead of 2-bromopropane, 1-bromopropane has recently come to be used as a new alternative. According to the report of the United Nations Environment Program (the Report of the Second Meeting of the Bureau of the Eleventh Meeting of the Parties to the Montreal Protocol on Substances that Deplete the Ozone Layer), the estimated sales of 1-bromopropane–based solvent for the period of January to December 1999 totaled 765 tons in Japan.

Our recent animal studies showed that 1-bromopropane could cause serious toxic effects on the central nervous system, peripheral nerves, and spermatogenesis (Ichihara *et al.*, 2000a, 2000b; Wang *et al.*, 2002; Yu *et al.*, 1998). While 1-bromopropane is less toxic to spermatogonia when compared with 2-bromopropane, it is known to inhibit spermiation in the testis (Ichihara *et al.*, 2000b). The underlying mechanism of the toxic effects of 1-bromopropane on the reproductive system is different from those of 2-bromopropane in male rats. On the other hand, previous study has revealed that a 10-week inhalation exposure to 1-bromopropane at 750 ppm reduced ovarian weight and numbers of corpora lutea, and also caused extended estrous cycle length (WIL Research Laboratories, 2001). The rats in the 500-ppm group displayed extended estrous cycle length without changing ovarian weight. Another study reported that exposure to 1-bromopropane increased relative weights of ovaries (Kim *et al*, 1999).

These studies suggested that 1-bromopropane might be a reproductive toxicant to not only male rats but also female rats. However, the former studies on female rats provided limited information on histopathological alterations of ovaries. It would be of great interest to characterize histopathological

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[1] To whom correspondence should be addressed. Fax: +81-52-744-2126. E-mail: tetsu@med.nagoya-u.ac.jp.

[2] Present address: Institute for Risk Analysis and Risk Communication, Department of Environmental Health, University of Washington School of Public Health and Community Medicine, 4225 Roosevelt Way NE #100, Seattle, WA 98105–6099.

[3] Present address: Research Center for Radiation Emergency Medicine, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan.

changes of ovaries or ovarian follicles induced by 1-bromopropane in comparison to the effects on primordial follicles by 2-bromopropane (Yu *et al.*, 1999), because previous studies on male rats demonstrated the difference of target in the testis between 1-bromopropane and 2-bromopropane (Ichihara *et al.*, 2000b; Omura *et al.*, 1997, 1999). The present study was designed to investigate the effects of 1-bromopropane on the female reproductive system, focusing on ovarian follicles, in rats.

MATERIALS AND METHODS

Chemical. 1-Bromopropane was kindly supplied by Tosoh Co., Ltd., Japan. Analytical analysis by capillary gas chromatography confirmed that the supplied stock was more than 99.5% pure.

Animals and inhalation exposure. Female Wistar rats, weighing 150–170 g at 10 weeks of age, were purchased from the Shizuoka Laboratory Animal Center, Japan. They were housed in stainless steel cages, provided food and water *ad libitum*, and kept on a 12-h/12-h light/dark cycle (lights on at 9 A.M.) at a constant temperature (23.0–25.0°C) and relative humidity (57–60%). The inhalation exposure system used in the present study has been described in detail previously (Huang *et al.*, 1989, 1990; Takeuchi *et al.*, 1989). The vapor concentration in the chamber was measured by gas chromatography and was digitally controlled within ±5% of the target concentration, using a personal computer system. Japanese laws concerning the protection and control of animals, standards related to care and management of experimental animals, and the Guide of Animal Experimentation of the Nagoya University Graduate School of Medicine were strictly followed throughout the study.

Experimental design. Forty female rats were divided into four equal groups. After monitoring estrous cycles for three weeks, each group was exposed daily to 0, 200, 400, or 800 ppm of 1-bromopropane by inhalation for eight h a day (from 2 to 10 P.M.), seven days a week. After exposure for 7 weeks, all rats of the 800-ppm group became seriously ill and were sacrificed by decapitation during the 8th week. Other groups were exposed for 12 weeks and decapitated on the day of diestrus I, during the 13th week.

Monitoring of estrous cycle. During the study period, daily vaginal smears were taken between 11:00 A.M. and 12:00 P.M. to monitor ovarian cycle pattern. The smears were stained with 0.5% methylene blue solution (Katayama Chemical, Japan) and examined under a light microscope. Cycle days were classified as proestrus, estrus, diestrus I, and diestrus II (Cooper *et al.*, 1993). For statistical analysis, the 15-week study period was divided into five consecutive 3-week periods: one preexposure period and four exposure periods. The estrous cycles were defined as normal when they showed typical stages of proestrus, estrus, and diestrus, which were usually observed with a four- to six-day cycle. When a cycle overlapped two consecutive periods, it was counted as a 0.5 cycle in each period. Estrous cycles were defined as regular cycles (3.0–5.0 cycles/3 weeks), irregular cycles (0.5–2.5 cycles/3 weeks), and no cycle (0 cycle/3 weeks).

Histopathological examination. Reproductive organs (right ovary, uterus, and vagina) and other organs (thymus, adrenal gland, kidney, spleen, liver, and brain) were dissected out carefully and weighed immediately. These organs, except the brain, were fixed in 10% neutral buffered formalin for light microscopic evaluation. Tissue blocks were embedded in paraffin and cut into 5-μm sections. They were mounted on glass slides, and stained with hematoxylin and eosin. Kidney tissue blocks were also stained by the PAS (Periodic Acid Schiff) method.

Counting of ovarian follicles. Serial sections (8-μm) were prepared from the left ovaries for counting follicles. A modified method of Pedersen and Peters (1968) for differential follicle counts was used according to Plowchalk *et al.* (1993), where types 1–3b, types 4–5b, and types 6–8 were grouped as primordial, growing, and antral follicles, respectively. Primordial follicles were included as oocytes with a complete one-layer ring of granulosa cells as well as oocytes devoid of such layers. Growing follicles were defined as oocytes, with multiple layers of surrounding granulosa cells without antrum formation. Antral follicles were defined as large oocytes with multiple layers of surrounding granulosa cells with fluid-filled antrum. No attempt was made to count atretic follicles or sum up total atresic follicle numbers. Follicles devoid of pyknotic nuclei, or that showed no chromatin redistribution, cytoplasmic condensation, or disarrangement of granulosa cells were considered as normal follicles. To avoid counting the same follicle that was large in diameter doubly, only follicles with explicit nucleoli on sections were counted. Follicles without visible nucleoli were not counted. Light microscopy was used for morphological characterization and maturation of follicles. Starting with the first serial section that contained the ovarian tissue, every fifth serial section was scored for differential follicle numbers. The numbers of each type of follicles in all sections were summed to determine the total primordial, growing, and antral ovarian follicle counts.

Hormonal assay. Blood plasma for hormonal assays was collected at decapitation. Concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined by a double-antibody radioimmunoassay by using rat LH and FSH RIA kits supplied by the National Hormone and Pituitary Program (Baltimore, MD). The values were expressed in terms of NIDDK-rLH-RP-3 and NIDDK-rFSH-RP-2, respectively (Maeda *et al.*, 1994; Tsukamura *et al.*, 1994). The lowest detection limits of LH and FSH were 0.16 ng/ml and 2.5 ng/ml, respectively. The intra-assay coefficient of variation for LH and FSH assay were 5.6 % for 51.1 ng/ml and 10.9 % for 13.7 ng/ml, respectively.

Statistical methods. Differences in body weight on every week, organ weight, and hormonal concentration between exposed groups and the control were analyzed by ANOVA followed by Dunnett's multiple comparison method. The number of ovarian follicles in each group was compared to the control by Dunnett's multiple comparison method following root transformation for normalizing each distribution. The data of organ weight, hormonal concentration, and the number of ovarian follicles were obtained, also from the 800-ppm group, but these data in this group were excluded in the statistical analysis. This is because there was not an appropriate age-matched control group for comparison. The number of estrous cycles in each group was compared to the control in each pre-exposure or exposure period by the Kruskal-Wallis test, followed by Dunnett-type multiple comparison method. A single rat from the control group was excluded from the analysis because extreme splenomegaly, hepatomegaly, and polycythemia were identified at autopsy. Data are expressed as mean ± SD. A p value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

The actual vapor concentrations of the exposed groups were 813 ± 28, 415 ± 19, and 200 ± 8 ppm, respectively. In the 800-ppm group, body weight was significantly decreased at each time point from 2 to 7 weeks after the beginning of exposure compared to the control, but the weight of the other two exposure groups did not significantly differ from the control (Fig. 1).

Vaginal smear examination showed that the number of normal estrous cycles in the 800-ppm group was significantly lower in the first exposure period compared with the control. Moreover, none of these rats had normal estrous cycles in the next exposure period. All anestrus rats displayed constant diestrus stage. In the 400-ppm group, the estrous cycles gradually prolonged around the 7–9th week of exposure, and all rats showing irregular estrous cycles displayed extended

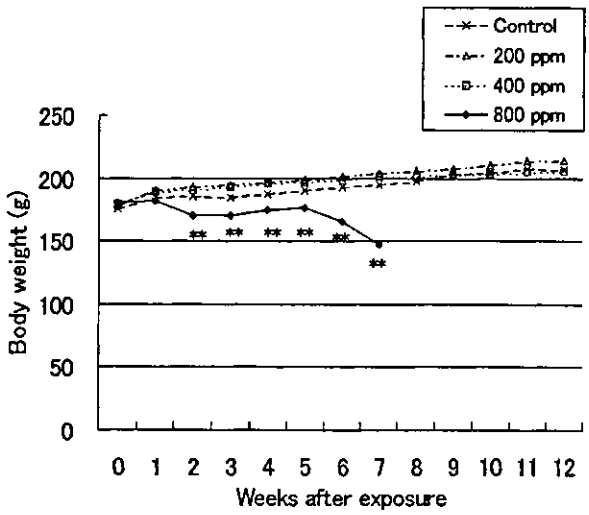

FIG. 1. Changes in body weight in rats exposed to 1-bromopropane. The rats in the 800-ppm group became seriously ill and were euthanized during the 8th week. Other groups were exposed to 1-bromopropane for 12 weeks. Body weight gain in the 800-ppm group was significantly less than the control, whereas the gain in rats of other groups did not significantly differ from the control. Differences in body weight were analyzed by ANOVA followed by Dunnett's multiple comparison method; $^{**}p < 0.01$.

diestrus. The number of normal estrous cycles was significantly lower in the last two periods. All anestrus rats also displayed constant diestrus smears in this group. In the 200-ppm group, there was no statistically significant change in estrous cycle compared to the control (Table 1).

The weight of reproductive organs in the 200- and 400-ppm groups was not significantly different from the control. The absolute weights of the liver and kidney were significantly higher in the 200- and 400-ppm groups compared with the control. The brain weights of the 400-ppm group were significantly lower than those of the control (Table 2 and Table 3).

Histopathological examination revealed abnormal findings in the examined organs only in the ovaries, kidneys, and liver. Normal growing and antral ovarian follicles in the 800-ppm group were observed less frequently than the control. In contrast, normal primordial follicles in the 800-ppm group were observed more frequently than in the control. No newly formed corpus luteum could be identified. The 400-ppm group showed reduced numbers of normal growing and antral ovarian follicles compared to the control, but the histopathological changes were milder than in the 800-ppm group. No significant change was found in the number of primordial follicles. In the 200-ppm group, histological examination of the ovary was almost normal, but antral follicle count was significantly less than in the control (Table 4, Fig. 2). Histopathological examination of the kidneys revealed mild dilatation of the proximal tubules in the 800-ppm group but no abnormal findings in the other exposure groups. In the 800-ppm group, scattered cytoplasmic degeneration was detected in hepatocytes located around the central veins in the liver, accompanied by nuclear pyknosis, but no serious changes, such as diffuse or focal necrosis, were noted. No abnormal findings were found in the livers of the other exposed groups.

Hormonal assays revealed no significant changes in plasma LH and FSH concentrations in the 200- and 400-ppm groups compared with the control (Table 5).

DISCUSSION

The major finding of the present study was that the disruption of estrous cycle in rats exposed to 400 ppm of 1-bromopropane was accompanied by decreases in numbers of growing and antral follicles. Exposure of the 200-ppm group also resulted in a significant decrease in the number of antral follicles, although such exposure did not cause disruption of the estrous cycle. These findings indicate that the number of antral follicles is the most sensitive indicator in evaluating

TABLE 1
Changes in Estrous Cycle in Rats Exposed to 1-Bromopropane by Inhalation

	Before exposure			1–3 weeks exposure			4–6 weeks exposure			7–9 weeks exposure			10–12 weeks exposure		
	Reg	Irreg	None	Reg	Irreg	None	Reg	Irreg	None	Reg	Irreg	None	Reg	Irreg	None
Control (n = 9)	9	0	0	9	0	0	9	0	0	9	0	0	9	0	0
200 ppm (n = 10)	10	0	0	9	1	0	10	0	0	10	0	0	10	0	0
400 ppm (n = 10)	10	0	0	10	0	0	9	0	1	7	3	0**	4	5	1**
800 ppm (n = 10)[a]	10	0	0	6	4	0*	0	5	5**						

Note. Reg, regular estrous cycles (3.0–5.0 cycles/3 weeks); Irreg, irregular estrous cycles (0.5–2.5 cycles/3 weeks), None, no normal estrous cycles (0 cycle/3 weeks). The number of estrous cycles in each group was compared to the control in each pre-exposure or exposure period by Dunnett-type multiple comparison method following Kruskal-Wallis test.

[a]Rats of the 800-ppm group became seriously ill and were euthanized by decapitation during the 8th week. Rats of the other groups were exposed to the agent for 12 weeks.

$^{*}p < 0.05$, $^{**}p < 0.01$.

TABLE 2
Body and Absolute Organ Weights at the End of the Experiment in Rats Exposed to 1-Bromopropane

	Control (n = 8)	200 ppm (n = 9)	400 ppm (n = 9)	800 ppm (n = 9)[a]
Ovary (R)	0.046 ± 0.006	0.048 ± 0.004	0.049 ± 0.009	0.026 ± 0.007
Ovary (L)	0.044 ± 0.006	0.045 ± 0.006	0.043 ± 0.009	0.023 ± 0.005
Ovary (R + L)	0.090 ± 0.005	0.093 ± 0.006	0.092 ± 0.016	0.049 ± 0.010
Uterus	0.462 ± 0.039	0.489 ± 0.044	0.433 ± 0.058	0.170 ± 0.017
Vagina	0.130 ± 0.010	0.146 ± 0.019	0.129 ± 0.016	0.142 ± 0.013
Thymus	0.159 ± 0.013	0.139 ± 0.026	0.144 ± 0.018	0.075 ± 0.021
Adrenal gland (R)	0.025 ± 0.002	0.028 ± 0.003	0.029 ± 0.003*	0.027 ± 0.004
Adrenal gland (L)	0.026 ± 0.002	0.029 ± 0.006	0.028 ± 0.003	0.028 ± 0.002
Adrenal gland (R + L)	0.051 ± 0.004	0.057 ± 0.008	0.056 ± 0.005	0.054 ± 0.005
Kidney (R)	0.731 ± 0.045	0.813 ± 0.045**	0.792 ± 0.043*	0.780 ± 0.035
Kidney (L)	0.742 ± 0.039	0.832 ± 0.046**	0.817 ± 0.035**	0.788 ± 0.059
Kidney (R + L)	1.473 ± 0.076	1.646 ± 0.081**	1.609 ± 0.077**	1.569 ± 0.091
Spleen	0.437 ± 0.026	0.462 ± 0.048	0.443 ± 0.027	0.294 ± 0.020
Liver	7.04 ± 0.47	8.27 ± 0.46**	9.20 ± 0.41**	7.94 ± 1.01
Brain	1.828 ± 0.024	1.778 ± 0.093	1.742 ± 0.037**	1.609 ± 0.028
Exposure duration	12 weeks	12 weeks	12 weeks	7 weeks
Body weight	207.3 ± 6.0	214.7 ± 7.9	205.2 ± 7.7	147.1 ± 8.2

Note. Body and absolute organ weights are expressed in grams. R, right; L, left. Differences in organ weights between the exposed groups and control were analyzed by ANOVA, followed by Dunnett's multiple comparison method.

[a]The rats in the 800-ppm group became seriously ill and were euthanized by decapitation during the 8th week. They were excluded from analysis because there was no appropriate age-matched control group for comparison (other groups were exposed for 12 weeks).

*$p < 0.05$, **$p < 0.01$.

female reproductive toxicity to 1-bromopropane in rats. Interestingly, the number of primordial follicles appeared to be larger in the 800-ppm group. Given the tendency for an increase in primordial follicles, along with the decrease in growing and antral follicles at higher exposure levels, the result might reflect at least part disruption of the follicular growth process. However, that might also reflect the difference of age at necropsy. The rats in the 800-ppm group were sacrificed at

TABLE 3
Relative Organ Weights at the End of the Experiment in Rats Exposed to 1-Bromopropane

	Control (n = 8)	200 ppm (n = 9)	400 ppm (n = 9)	800 ppm (n = 9)[a]
Ovary (R)	0.22 ± 0.03	0.23 ± 0.02	0.24 ± 0.05	0.18 ± 0.04
Ovary (L)	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.05	0.15 ± 0.03
Ovary (R + L)	0.44 ± 0.03	0.43 ± 0.04	0.45 ± 0.09	0.33 ± 0.06
Uterus	2.23 ± 0.19	2.23 ± 0.20	2.11 ± 0.27	1.16 ± 0.11
Vagina	0.63 ± 0.04	0.68 ± 0.10	0.63 ± 0.07	0.96 ± 0.06
Thymus	0.77 ± 0.07	0.65 ± 0.12	0.70 ± 0.09	0.51 ± 0.13
Adrenal gland (R)	0.12 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.18 ± 0.03
Adrenal gland (L)	0.12 ± 0.01	0.14 ± 0.03	0.14 ± 0.01	0.19 ± 0.02
Adrenal gland (R + L)	0.24 ± 0.02	0.27 ± 0.04	0.27 ± 0.02	0.37 ± 0.04
Kidney (R)	3.53 ± 0.20	3.79 ± 0.14**	3.89 ± 0.14**	5.31 ± 0.17
Kidney (L)	3.58 ± 0.18	3.88 ± 0.18**	3.98 ± 0.12**	5.36 ± 0.22
Kidney (R + L)	7.11 ± 0.33	7.67 ± 0.26**	7.84 ± 0.25**	10.67 ± 0.33
Spleen	2.11 ± 0.10	2.15 ± 0.21	2.16 ± 0.13	2.00 ± 0.08
Liver	33.9 ± 1.6	38.5 ± 1.4**	44.8 ± 1.4**	53.8 ± 4.3
Brain	8.83 ± 0.31	8.29 ± 0.48	8.50 ± 0.38	10.97 ± 0.52
Exposure duration	12 weeks	12 weeks	12 weeks	7 weeks

Note. Relative organ weights are expressed in mg tissue weight/g body weight. R, right; L, left. Differences in organ weights between the exposed groups and control were analyzed by ANOVA, followed by Dunnett's multiple comparison method.

[a]The rats in the 800-ppm group became seriously ill and were euthanized by decapitation during the 8th week. They were excluded from analysis because there was no appropriate age-matched control group for comparison (other groups were exposed for 12 weeks).

*$p < 0.05$, **$p < 0.01$.

TABLE 4
Number of Normal Ovarian Follicles in Rats Exposed to 1-Bromopropane

	Control (n = 8)	200 ppm (n = 9)	400 ppm (n = 9)	800 ppm (n = 9)[a]
Primordial	176.8 ± 48.8	157.8 ± 49.4	206.0 ± 66.6	423.1 ± 140
Growing	70.0 ± 20.3	53.4 ± 17.9	47.2 ± 17.3*	30.1 ± 15.1
Antral	30.1 ± 22.4	12.6 ± 4.82*	7.44 ± 6.52**	3.78 ± 3.87
Exposure duration	12 weeks	12 weeks	12 weeks	7 weeks

Note. The number of ovarian follicles in each group was compared to the control by Dunnett's multiple comparison method, following root transformation for normalizing each distribution.

[a]The rats in the 800-ppm group became seriously ill and were euthanized by decapitation during the 8th week. They were excluded from analysis because there was no appropriate age-matched control group for comparison (other groups were exposed for 12 weeks).

*$p < 0.05$, **$p < 0.01$.

21 weeks of age although other groups were in necropsy at 26 weeks of age. Younger rats would have larger numbers of primordial follicles than older rats, since primordial follicles could not be created in adult rats.

2-Bromopropane, an isomer of 1-bromopropane, also causes disruption of the estrous cycles and the disappearance of such cycles following long-term exposure in nonpregnant female rats exposed to 1000 ppm (Kamijima *et al.*, 1997). In another study, the same dose was found to decrease the number of ovarian follicles of all stages including primordial follicles (Yu *et al.*, 1999). These results suggest that 2-bromopropane may target primordial follicles. Several chemicals exhibit toxic effects on primordial follicles such as 9,10-dimethylbenzanthracene, 3-methylcholanthrene, benzo[*a*]pyrene, 4-vinylcyclohexene, and its diepoxide metabolite (Borman *et al.*, 2000). In this regard, a decrease in the number of primordial follicles could cause early menopause.

In contrast to the above findings on 2-bromopropane, 1-bromopropane seems to mainly alter the maturation of follicles and is less toxic to follicles at early stages. A number of chemical compounds are known to impair ovarian follicle maturation. For example, at high dose, ethinylestradiol increased the number of apoptotic corpora lutea and early-stage ovarian follicles (Andrews *et al.*, 2002). Methoxychlor induced atrophy of the ovary, accompanied by degeneration of small and antral follicles and complete atrophy of the corpora lutea (Chapin *et al.*, 1997). These effects may be due to inhibition of the hypothalamic-pituitary-ovarian axis (Sarkar *et al.*, 1989).

The above differences between the toxic effects of 1-bromopropane and 2-bromopropane on female reproductive organs seem to parallel the differences in the actions of the two isomers on the male reproductive system. Previous studies showed that long-term exposure to 2-bromopropane resulted in reductions in the numbers of all types of spermatogenic cells (Ichihara *et al.*, 1997) and that acute or subacute exposure impaired spermatogenesis (Omura *et al.*, 1999), followed by apoptosis of spermatocytes (Yu *et al.*, 2001). On the other hand, 1-bromopropane had little effect on spermatogonia, spermatocytes, and round spermatid (Ichihara *et al.*, 2000b), and only a mild effect on weight gain of seminal vesicles and a failure of spermiation in seminiferous tubules in male rats (Ichihara *et al.*, 2000b). Considered together, these results indicate that 1-bromopropane has adverse effects on both male and female reproductive organs, although the mechanisms of these effects are different from those of 2-bromopropane.

It is well known that 1-bromopropane also exhibits severe neurotoxicity (Ichihara *et al.*, 2000; Yu *et al.*, 1998). Sclar (1999) reported a case of an intoxicated male worker who developed encephalomyeloradiculoneuropathy following exposure to 1-bromopropane. Recently, Ichihara *et al.* (2002) reported three female cases of 1-bromopropane toxicity. Because one of these cases was a 50-year-old female, she had menopause before exposure to 1-bromopropane. However, the remaining two females showed temporal disruption of the estrous cycle. Thus, 1-bromopropane may cause severe toxicity in humans, including reproductive and neurological dysfunction, and may impair the central nervous system as well as the peripheral nerves.

The adverse effects of 1-bromopropane on the ovaries may be indirect through the disruption of hormonal regulation following central nervous system impairment. It is known that suppression of pulsatile luteinizing hormone secretion impairs ovarian follicle maturation (Maeda *et al.*, 1994; Tsukamura *et al.*, 1994). However, our results in sex hormonal assays showed no significant changes. LH and FSH concentrations may not be good indicators of endocrine regulation since the levels of these hormones are to a large extent influenced by estrous cycle and pulsatile secretion. In addition, the present study did not investigate the effect on the surging level of hormones. Studies are currently underway in our laboratories to examine the effect of 1-bromopropane on pulsatile gonadotropin secretion and surging level.

Compared to monitoring of the estrous cycle or fertility, counts of ovarian follicle differentiation may be the most sensitive quantitative indicator of female reproductive toxicity and could predict the type of reproductive disruption that may be caused by exposure to chemicals (Bolon *et al.* 1997; Yu *et al.*, 1999). However, only limited work is available in which follicle counting was used in serial sections (Bolon *et al.*, 1997; Borman *et al.*, 2000; Yu *et al.*, 1999). The present study

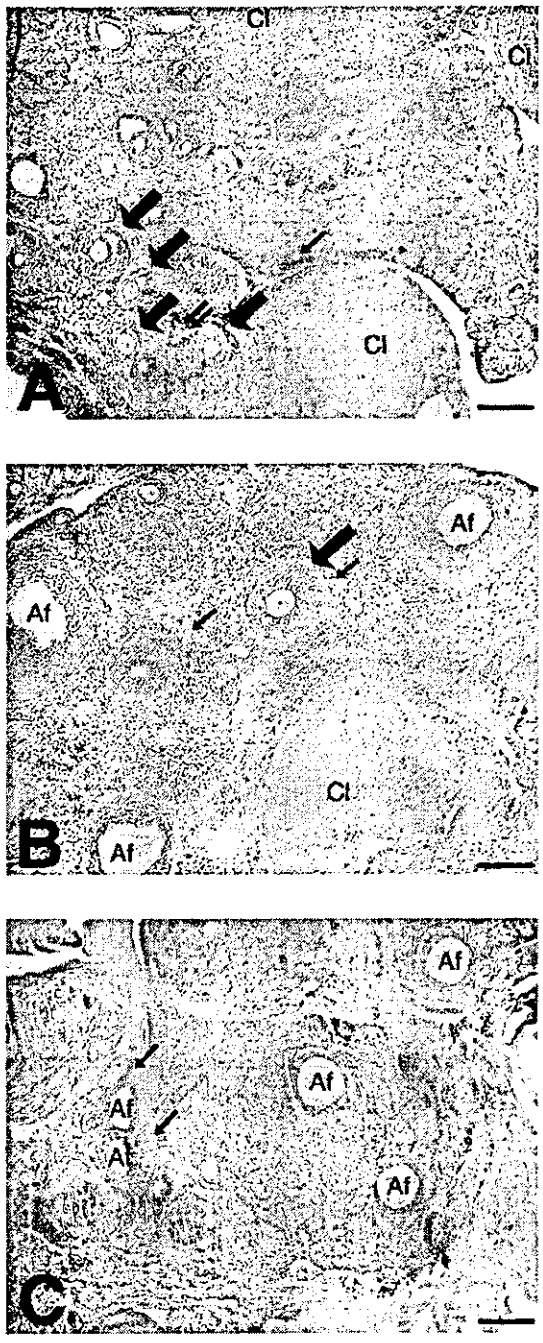

FIG. 2. Histopathological examination of ovaries of control rats and those exposed to 1-bromopropane. Small arrows: normal primordial follicles. Large arrows: normal growing follicles. (A) Control group. Most of the ovarian follicles and corpora lutea are normal; (B) 400-ppm group. Note the decrease in normally growing and antral ovarian follicles. However, these changes are milder than those of the 800-ppm group; (C) 800-ppm group. Note that most of the ovarian follicles are atretic. Note also the lack of fresh corpus luteum. Cl: corpus luteum, Af: atretic follicle. Bar, 200 μm. (Hematoxylin-eosin staining; original magnification ×100).

showed a significant decrease in antral follicles in the 200-ppm group, though there was no significant change in the estrous cycle in this group. This finding supports the notion that ovarian follicle count may be the most sensitive indicator of female reproductive dysfunction.

Our results demonstrated significant increases in the absolute weights of kidney and liver in the 200- and 400-ppm groups without any changes in body weight. Mild dilation of proximal tubules observed at 800 ppm might relate to increase in kidney weight, but such histopathological change is not clear at lower levels. Dilation of proximal tubules may suggest tubular dysfunction (Hanley, 1980; Olsen *et al.*, 1993), but the present study lacks the data of biological markers of tubular function, such as *N*-acetylglutamate. There are also no qualitative histopathological alterations explaining weight gain in liver. However, a possibility of microsomal induction might be considered, as liver weight gain usually accompanied hepatic microsomal induction in the rats administered many kind of organic solvents (Nakajima *et al.*, 1991). Weight gain in adrenal glands at 400-ppm dosage might suggest stress reaction involved with the central nervous system. On the other hand, male rats did not show such a weight change in kidney or adrenal gland in any exposed groups, although the absolute weight of liver increased at 800-ppm doses (Ichihara *et al.*, 2000b). These results might reflect a sex difference in the sensitivity of kidneys to 1-bromopropane. Increase in liver or kidney weights was not observed in male (Ichihara *et al.*, 1997) or female rats (Kamijima *et al.*, 1997) exposed to 2-bromopropane; rather, decrease in kidney and liver weights were observed in male rats exposed to 2-bromopropane (Ichihara *et al.*, 1997). These phenomena also might suggest a difference of toxic mechanism between 1-bromopropane and 2-bromopropane.

A significant absolute brain weight loss, which was detected in our females rats exposed to 400 ppm of 1-bromopropane, was also observed in male rats exposed to 800 ppm (Ichihara *et al.*, 2000a). In this regard, toluene is a well-known neurotoxic agent but it never results in a decrease in brain weight in rats, even after exposure to 1000 ppm (Huang *et al.*, 1990). These findings highlight the severity of neurotoxicity of 1-bromopropane.

In conclusion, we have demonstrated in the present study that 1-bromopropane impaired female reproductive functions in rats in a manner different from that of 2-bromopropane. We emphasize the need for careful handling of this chemical compound in the industry.

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TABLE 5

Plasma Concentrations of Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) in Rats Exposed to 1-Bromopropane

	Control (n = 8)	200 ppm (n = 9)	400 ppm (n = 9)	800 ppm (n = 9)[a]
LH (ng/ml)	0.28 ± 0.29	0.27 ± 0.23	0.19 ± 0.21	0.28 ± 0.44
FSH (ng/ml)	8.10 ± 1.58	8.33 ± 1.49	8.79 ± 1.49	11.2 ± 1.49
Exposure duration	12 weeks	12 weeks	12 weeks	7 weeks

Note. Differences in plasma hormone levels between various exposure groups and the control were analyzed by ANOVA, followed by Dunnett's multiple comparison method.

[a]The rats in the 800-ppm group became seriously ill and were euthanized by decapitation during the 8th week. They were excluded from analysis because there was no appropriate age-matched control group for comparison (other groups were exposed for 12 weeks).

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Dose-Dependent Biochemical Changes in Rat Central Nervous System after 12-Week Exposure to 1-Bromopropane

Hailan Wang[1,*], Gaku Ichihara[1], Hidenori Ito[2], Kanefusa Kato[2], Junzoh Kitoh[3], Tetsuya Yamada[1], Xiaozhong Yu[1], Seiji Tsuboi[4], Yoshinori Moriyama[4], Yasuhiro Takeuchi[1,5]

[1]*Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi, Japan*
[3]*Nagoya University Graduate School of Medicine, Nagoya, Japan*
[4]*Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan*
[5]*Research Center for Radiation Emergency Medicine, National Institute of Radiological Sciences, Chiba, Japan*

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Abstract

1-Bromopropane is used as a cleaning agent or adhesive solvent in the workplace. The present study investigated the long-term effects of exposure to 1-bromopropane on biochemical components in the central nervous system (CNS) of rats. Four groups, each of nine male Wistar rats, were exposed to 200, 400, or 800 ppm 1-bromopropane or fresh air only, 8 h per day, 7 days a week for 12 weeks. We measured the levels of neuron-specific γ-enolase, glia-specific β-S100 protein, creatine kinase (CK) subunits B and M, heat shock protein Hsp27 (by enzyme immunoassay), enzymatic activity of CK and levels of glutathione (GSH), oxidized glutathione (GSSG) and sulfhydrul (SH) base in the cerebrum, cerebellum, brainstem and spinal cord. γ-Enolase decreased dose-dependently in the cerebrum, which showed a decrease in wet weight, at 400 ppm or over, but no change was noted in β-S100 protein in any brain region or spinal cord. Hsp27 decreased in the cerebellum, brainstem and spinal cord. Protein-bound SH base, non-protein SH base and total glutathione decreased in every brain region. CK activity decreased dose-dependently at 200 ppm or over, and the ratio of CK activity to CK-B concentration tended to decrease in all regions. The decrease in γ-enolase in the cerebrum suggests the involvement of biochemical changes in neurons with decrease in the wet weight of the cerebrum. Glutathione depletion and changes in proteins containing SH base as a critical site might be the underlying neurotoxic mechanism of 1-bromopropane. The biochemical changes in the cerebrum indicate that long-term exposure to 1-bromopropane has effects on the CNS.

Keywords: 1-Bromopropane; γ-Enolase; Creatine kinase; Glutathione; Sulfhydryl

INTRODUCTION

The compound 1-bromopropane is an alternative to ozone-depleting solvents. It is now used as a cleaning agent or adhesive solvent in workplaces, because of its lower ozone-depleting potency, high volatility and noninflammability. However, we detected adverse effects for 1-bromopropane on the nervous system in rats (Yu et al., 1998; Ichihara et al., 2000). Rats exposed to 1-bromopropane for 12 weeks exhibited decrease in limb muscle strength, electrophysiological and morphological changes in the peripheral nerves,

* Corresponding author. Tel.: +81-52-744-2124; fax: +81-52-744-2126.
E-mail address: wanghl@med.nagoya-u.ac.jp (H. Wang).

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and swelling of preterminal axons in the gracile nucleus of the medulla oblongata. Exposure to higher concentrations resulted in a decrease in the wet weight of the cerebrum and pyknotic degeneration of cerebellar Purkinje cells. However, animal studies provided limited morphological evidence as to the central nervous system (CNS), compared to the peripheral nerves. We suspected possible adverse effects of 1-bromopropane on the CNS because of the following three findings. (1) The decrease in the wet weight of the cerebrum was found in the 12-week study (Ichihara et al., 2000). This change was surprising because it was not observed even in rats exposed to toluene, which is known to cause cerebral atrophy in sniffing humans (Antti-Poika, 1987; Ron, 1986). (2) Previous animal studies on toluene toxicity showed that the changes in glia and neuron-specific protein correlated with the clinical features in affected humans (Huang et al., 1990, 1992), although little morphological evidence was noted in rats exposed to toluene at realistic exposure levels and duration (Bruckner and Peterson, 1981; Ladefoged et al., 1991). The latter does not necessarily exclude possible adverse effects, especially with respect to the CNS, because morphological changes in the CNS would be more robust than biochemical changes seen in neurotoxicity. (3) Characterization of the biochemical changes might allow the identification of the underlying mechanism of toxicity. Thus, we examined the CNS of rats and determined the biochemical changes in the last 7 days of the study following exposure to 1-bromopropane, and detected changes in few biochemical indices such as γ-enolase and glutathione (Wang et al., 2002). However, the study had certain limitations because it did not examine the long-term effects of 1-bromopropane and the changes were suspected to represent the acute response to exposure. In the present study, we investigated the biochemical changes in the CNS following 12-weeks of exposure to 1-bromopropane. We measured changes in neuron-specific γ-enolase, glia-specific β-S100 protein and heat shock protein of 25–28 kDa (Hsp27) following exposure to determine the vulnerable regions in the CNS. We also measured creatine kinase (CK) activity and the concentrations of isozymes creatine kinase-BB (CK-B) and creatine kinase-MM (CK-M) in the CNS, based on our observation of reduced CK activity in the plasma and CNS in 12-week and 7-day preliminary studies, respectively. Glutathione and sulfhydryl (SH) base levels were also evaluated because biomolecules containing sulfhydryl base could be targets of 1-bromopropane, as shown by conjugation of 1-bromopropane with glutathione in rats (Jones and Walsh, 1979; Barnsley et al., 1966).

MATERIALS AND METHODS

Animal and Exposure

Thirty-six specific pathogen-free 9-week-old male Wistar rats (body weight 240–260 g) were purchased from the Shizuoka Laboratory Animal Center, Japan. They were housed and acclimatized to their new environment for one week, and were then randomly divided into four equal groups, each of nine rats. They were housed in a 12:12 light:dark cycle (lights on at 9:00 a.m. and off at 9:00 p.m.), stable relative humidity (57–60%) and constant temperature (23–25 °C) room. Food and water were provided ad libitum.

The four groups of rats were exposed to 200, 400 or 800 ppm 1-bromopropane or fresh air, respectively, in inhalation chambers for 8 h a day, 7 days a week for 12 weeks. Daily exposure commenced at 2:00 p.m. and was terminated at 10:00 p.m. The inhalation exposure system has been described previously (Takeuchi et al., 1989; Ichihara et al., 1997). The vapor concentration in the chamber was measured every 10 s with gas chromatography and controlled to within ±5% of the target concentration with a personal computer. After 12 weeks of exposure, the measured 1-bromopropane gas concentrations in the three chambers were 208 ± 15, 412 ± 24, and 821 ± 38 ppm (mean ± S.D.), respectively. 1-Bromopropane (99.81% purity) was supplied by Tosoh Co., Japan.

These experiments were conducted in accordance with the Japanese law concerning the protection and control of animals and the Guidelines for Animal Experimentation of the Nagoya University Graduate School of Medicine.

Animal Necropsy

Rats were sacrificed at 12–16 h after the end of the last exposure to 1-bromopropane. The necropsy was conducted in ascending and descending orders alternately to prevent bias of necropsy order. After exsanguinations through the abdominal aorta under pentobarbital anesthesia, the brain and spinal cord (2 cm above the last thoracic vertebra, which corresponds with the anterior lumbar enlargement) were rapidly removed. The brain was immediately dissected including the cerebrum without olfactory bulb (containing the neocortex, hippocampus, piriform cortex, amygdaloid nuclei, caudate putamen, globus pallidus and olfactory tubercle), cerebellum and brainstem (including medulla oblongata, pons, midbrain and thalamus) on a steel plate placed on ice. The cerebellum

was separated by cutting the cerebellar peduncles. Brain and spinal cord tissue blocks were weighed and kept frozen at −80 °C until analysis.

Immunoassay of Nerve-Specific Marker Proteins

Tissue blocks of the cerebrum, cerebellum, brainstem and the spinal cord were homogenized in 10 volumes (w/v) of 100 mM citrate buffer (pH 7.4) containing 20 mM EDTA at 0 °C. The homogenate was centrifuged at 45,000 × *g* for 20 min at 4 °C. The supernatant was used for analysis of nerve-specific proteins, CK activity and estimation of soluble protein concentrations. Neuronal marker protein γ-enolase, glial cell marker protein β-S100 protein, CK-B, CK-M and Hsp27 were determined by the highly sensitive sandwich-type enzyme immunoassay system developed by Kato et al. (1981, 1982, 1986, 1999). Protein concentrations of the soluble fractions of homogenates were estimated by the dye-binding method of Bradford (1976) using Bio-Rad (Hercules, CA) reagents.

Quantitative Analysis of Total Glutathione (Total-GSH), Oxidized Glutathione (GSSG), Protein-Bound Sulfhydryl Groups (PSH) and Non-Protein-Bound Sulfhydryl Groups (NSH)

Protein-bound and non-protein-bound sulfhydryl groups were quantified essentially as described by Habeeb (1972). The homogenized cerebrum, cerebellum, brainstem and spinal cord were treated with trichloroacetic acid solution at a final concentration of 5%, and then pelleted by centrifugation at 15,000 × *g* for 20 min. Each pellet was dissolved in 40 mM NaPB buffer (pH 8.0) containing 1%SDS and 0.025%EDTA. Sulfhydryl groups in the pellet (protein-bound sulfhydryl base, PSH) and the supernatants (non-protein-bound sulfhydryl base, NSH) were quantified by incubation with 5,5′-dithiobis-(2-nitrobenzoic) (DTMB) in 80 mM NaPB buffer (pH 8.0) containing 2%SDS and 0.05%EDTA at room temperature. The supernatant was also used for quantification of total and oxidized glutathione by the method developed by Matsumoto et al. (1996). For total glutathione determination, the supernatant was diluted 100 times with 125 mM sodium phosphate (pH 7.5) containing 6.3 mM EDTA. An aliquot (50 μl) of the diluted solution was assayed at 412 nm in a 1 ml mixture comprising 0.21 μM NADPH, 0.6 μM DTMB, 125 μM sodium phosphate (pH 7.5) and 6.3 μM EDTA. For GSSG determination, 20 μl of commercially available acrylonitrile (final concentration 295 mM), 50–200 μl of the supernatant and 125 μM sodium phosphate (pH 8.0) containing 6.3 μM EDTA were mixed to a final volume of 1 ml, and were incubated at 25 °C for 10 min. An aliquot of the preincubation mixture was assayed.

Statistical Analysis

Data were expressed as mean ± S.D. Multiple comparisons between the exposure groups and the control were tested using Dunnett's method following one-way analysis of variance (ANOVA). A probability $P < 0.05$ denoted the presence of a significant statistical difference.

RESULTS

Rats exposed for 12 weeks to 800 ppm 1-bromopropane showed a significant decrease in the wet weight of the cerebrum, and those exposed to 400 and 800 ppm for the same period showed a significant decrease in body weight (Table 1). Exposure to 1-bromopropane resulted in a dose-dependent decrease in tissue concentrations of γ-enolase in the cerebrum, which was significant at both 400 and 800 ppm. No significant changes were noted in β-S100 protein levels in any regions of the CNS. Significantly higher levels of

Table 1
Body and brain weights in rats exposed to 1-bromopropane for 12 weeks[a]

	Control ($n = 9$)	200 ppm ($n = 9$)	400 ppm ($n = 9$)	800 ppm ($n = 9$)
Body weight (g)	432 ± 21	426 ± 25	403 ± 25*	382 ± 16*
Absolute organ weights				
Cerebrum (g)	1.14 ± 0.03	1.13 ± 0.03	1.11 ± 0.03	1.05 ± 0.04*
Cerebellum (g)	0.28 ± 0.01	0.28 ± 0.01	0.27 ± 0.02	0.27 ± 0.01
Brainstem (g)	0.64 ± 0.04	0.62 ± 0.02	0.63 ± 0.04	0.6 ± 0.04

[a] Values are mean ± S.D.
* $P < 0.05$, compared with the control, by Dunnett's multiple comparison.

Table 2
γ-Enolase, β-S100 protein and Hsp27 in rat brain and spinal cord tissues following 12-weeks of exposure to 1-bromopropane[a]

	Control ($n = 9$)	200 ppm ($n = 9$)	400 ppm ($n = 9$)	800 ppm ($n = 9$)
γ-Enolase (ng/μg soluble protein)				
Cerebrum	7.68 ± 1.27	6.03 ± 1.49	5.82 ± 1.48*	5.71 ± 1.43*
Cerebellum	29.89 ± 2.16	29.93 ± 2.30	29.96 ± 1.97	29.22 ± 2.00
Brainstem	4.80 ± 1.16	4.88 ± 0.81	4.08 ± 0.78	4.72 ± 0.93
Spinal cord	2.05 ± 0.52	2.14 ± 0.41	2.13 ± 0.69	2.42 ± 0.72
β-S100 (ng/μg soluble protein)				
Cerebrum	2.44 ± 0.24	2.32 ± 0.28	2.70 ± 0.36	2.52 ± 0.48
Cerebellum	3.30 ± 0.09	3.10 ± 0.43	3.08 ± 0.72	3.33 ± 0.61
Brainstem	3.77 ± 0.47	4.20 ± 0.93	4.38 ± 0.68	4.49 ± 0.44
Spinal cord	7.39 ± 1.21	6.50 ± 0.81	9.23 ± 1.83	7.93 ± 0.95
Hsp27 (pg/μg soluble protein)				
Cerebrum	44.09 ± 5.70	46.12 ± 10.7	39.82 ± 5.30	42.25 ± 7.80
Cerebellum	34.54 ± 4.50	36.31 ± 4.50	35.86 ± 4.50	44.37 ± 5.2*
Brainstem	126.38 ± 21.10	144.54 ± 26.2	151.54 ±18.40	190.61 ± 41.2*
Spinal cord	433.51 ± 77.10	446.16 ± 54.9	536.63 ± 94.00	704.65 ± 199.5*

[a] Values are mean ± S.D.
* $P < 0.05$, compared with the control, by Dunnett's multiple comparison.

Hsp27 were noted in the cerebellum, brainstem and spinal cord of rats exposed to 1-bromopropane at 800 ppm (Table 2).

CK activity decreased in a dose-dependent fashion, and to a significant extent in the cerebrum, cerebellum, brainstem and spinal cord in all exposed groups (Table 3). CK-B concentrations decreased significantly in the cerebrum, cerebellum and brainstem at 800 ppm, and CK-B decreased dose-dependently in the spinal cord in all exposure groups. However, the ratios of CK activity/CK-B concentration decreased in the cerebrum, brainstem and spinal cord. CK-M tissue concentrations decreased significantly only in the brainstem of rats exposed to 1-bromopropane at 400 and 800 ppm (Table 4).

Total glutathione concentrations were significantly lower in the cerebrum, cerebellum and brainstem of rats treated with 800 ppm 1-bromopropane than in the controls, but were significantly higher in the spinal cord after exposure to 800 ppm than in controls. GSSG increased dose-dependently only in the cerebrum, while NSH significantly decreased in both the cerebrum and brainstem of rats exposed to 800 ppm 1-bromopropane and PSH significantly decreased in the cerebellum in both the 400 and 800 ppm exposure groups, and in the brainstem in the 800 ppm group (Table 5). No significant changes were observed in concentrations of total soluble protein in the brain and spinal cord in exposed rats (Table 6).

DISCUSSION

Rats exposed for 12 weeks to 1-bromopropane showed a significant decrease in neuron-specific γ-enolase in the cerebrum, but not in the cerebellum. This change paralleled the decrease in the wet weight of the cerebrum. The specific decrease in neuron-specific γ-enolase in the cerebrum suggests the involvement of biochemical changes in neurons with the

Table 3
CK activities in rat brain and spinal cord tissues after 12-weeks of exposure to 1-bromopropane[a]

	Control ($n = 9$)	200 ppm ($n = 9$)	400 ppm ($n = 9$)	800 ppm ($n = 9$)
CK activity (IU/mg soluble protein)				
Cerebrum	439.9 ± 21.4	395.6 ± 29.7	317.3 ± 54.5*	243.0 ± 18.7*
Cerebellum	599.6 ± 24.2	538.7 ± 68.5	515.2 ± 31.3*	381.3 ± 27.0*
Brainstem	472.4 ± 32.6	395.9 ± 35.1	334.6 ± 13.4*	250.4 ± 16.7*
Spinal cord	446.6 ± 29.9	371.0 ± 39.2*	302.6 ± 19.2*	210.2 ± 23.9*

[a] Values are mean ± S.D.
* $P < 0.05$, compared with the control, by Dunnett's multiple comparison.

Table 4
CK-B and CK-M levels in rat brain and spinal cord tissues after 12-weeks of exposure to 1-bromopropane[a]

	Control ($n = 9$)	200 ppm ($n = 9$)	400 ppm ($n = 9$)	800 ppm ($n = 9$)
CK-B (ng/μg soluble protein)				
Cerebrum	11.78 ± 1.82	12.35 ± 4.62	11.57 ± 1.93	8.21 ± 1.07*
Cerebellum	20.12 ± 2.91	20.51 ± 2.38	17.95 ± 2.58	13.69 ± 1.86*
Brainstem	11.78 ± 1.25	11.28 ± 2.05	10.89 ± 1.46	8.43 ± 1.33*
Spinal cord	9.17 ± 1.05	6.83 ± 1.40*	6.55 ± 1.33*	5.61 ± 0.59*
CK-M (pg/μg soluble protein)				
Cerebrum	7.32 ± 3.83	6.83 ± 2.63	7.13 ± 3.65	6.24 ± 2.69
Cerebellum	4.89 ± 2.80	4.59 ± 1.80	5.37 ± 2.10	4.77 ± 2.90
Brainstem	12.64 ± 5.30	8.89 ± 3.60	7.66 ± 2.64*	8.19 ± 2.5*
Spinal cord	66.53 ± 9.38	49.31 ± 9.38	55.54 ± 9.38	31.74 ± 9.38
CK activity/CK-B				
Cerebrum	41.70 ± 9.95	35.00 ± 9.92	26.80 ± 6.4*	28.60 ± 2.1*
Cerebellum	32.80 ± 8.00	26.90 ± 4.50	28.00 ± 4.50	27.20 ± 3.70
Brainstem	42.20 ± 10.40	37.60 ± 6.00	30.20 ± 4.5*	29.10 ± 5.7*
Spinalcord	52.40 ± 9.90	53.30 ± 15.60	47.60 ± 9.70	38.30 ± 7.1*

[a] Values are mean ± S.D.
* $P < 0.05$, compared with the control, by Dunnett's multiple comparison.

reduction in wet weight of the cerebrum. On the other hand, glia-specific β-S100 protein was not enhanced in any regions of the CNS after 12-weeks of exposure. The responses of γ-enolase and β-S100 protein to 1-bromopropane exposure were different from these reported following toluene exposure, which increased both γ-enolase and β-S100 protein in the cerebellum (Huang et al., 1990, 1992). The present findings of marker proteins might be characteristic for 1-bromopropane. In the cerebellum, no changes were noted in γ-enolase and β-S100 protein following 12-weeks of exposure. These results suggest that the decrease

Table 5
GSH, GSSG, NSH and PSH levels in rat brain and spinal cord tissues after 12-weeks of exposure to 1-bromopropane[a]

	Control ($n = 9$)	200 ppm ($n = 9$)	400 ppm ($n = 9$)	800 ppm ($n = 9$)
Total-GSH (nmol/g brain tissue)				
Cerebrum	955.2 ± 77.4	986.9 ± 82.2	997.4 ± 65.7	781.3 ± 70.2*
Cerebellum	860.5 ± 50.9	871.4 ± 31.5	846.6 ± 50.8	714.9 ± 105.8*
Brainstem	653.4 ± 57.8	693.8 ± 70.2	712.5 ± 47.4	561.7 ± 57.1*
Spinal cord	389.0 ± 15.6	414.2 ± 25.9	416.2 ± 31.2	498.7 ± 49.6*
GSSG (nmol/g brain tissue)				
Cerebrum	19.2 ± 10.2	23.0 ± 4.2	25.8 ± 3.8	35.7 ± 13.5*
Cerebellum	14.4 ± 4.3	17.2 ± 4.4	14.2 ± 4.2	16.4 ± 4.8
Brainstem	18.2 ± 4.6	19.5 ± 2.5	16.2 ± 2.4	15.6 ± 2.1
Spinal cord	29.5 ± 5.5	31.0 ± 5.1	30.7 ± 9.6	29.2 ± 6.8
NSH (μmol/g brain tissue)				
Cerebrum	1.61 ± 0.10	1.59 ± 0.04	1.56 ± 0.10	1.29 ± 0.10*
Cerebellum	1.59 ± 0.08	1.61 ± 0.09	1.68 ± 0.18	1.68 ± 0.14
Brainstem	1.17 ± 0.08	1.16 ± 0.10	1.23 ± 0.08	1.03 ± 0.05*
Spinal cord	0.88 ± 0.07	0.85 ± 0.06	0.91 ± 0.06	0.85 ± 0.11
PSH (μmol/g soluble protein)				
Cerebrum	97.51 ± 8.34	93.88 ± 6.25	96.72 ± 4.54	90.73 ± 5.86
Cerebellum	109.92 ± 7.33	105.45 ± 7.72	96.73 ± 3.82*	102.36 ± 4.79*
Brainstem	117.65 ± 8.90	120.47 ± 7.71	120.47 ± 4.60	107.45 ± 3.87*
Spinal cord	106.69 ± 16.87	113.96 ± 12.35	101.97 ± 10.03	98.34 ± 10.67

[a] Values are mean ± S.D.
* $P < 0.05$, compared with the control, by Dunnett's multiple comparison.

Table 6
Concentrations of total soluble protein in rat brain and spinal cord after 12-weeks of exposure to 1-bromopropane[a]

	Control ($n = 9$)	200 ppm ($n = 9$)	400 ppm ($n = 9$)	800 ppm ($n = 9$)
Soluble protein (mg/g wet tissue)				
Cerebrum	31.18 ± 2.18	31.15 ± 3.32	30.47 ± 3.57	32.47 ± 1.73*
Cerebellum	29.90 ± 2.16	29.98 ± 2.30	29.96 ± 1.97	29.22 ± 2.00
Brainstem	27.36 ± 1.47	26.90 ± 1.62	27.14 ± 1.56	26.14 ± 3.22
Spinal cord	20.72 ± 1.83	21.21 ± 1.74	20.49 ± 1.11	20.78 ± 1.10

[a] Values are mean ± S.D.
* $P < 0.05$, compared with the control, by Dunnett's multiple comparison.

in γ-enolase in the cerebellum observed in the 7-day exposure study (Wang et al., 2002) probably represented a transient effect of 1-bromopropane. Histological studies also did not show any abnormality in the cerebellum of rats exposed to 1-bromopropane at 800 ppm or less (Ichihara et al., 2000), although exposure at a higher level of 1000 ppm induced pyknotic shrinkage of Purukinje cells along with systemic debilitation (Yu et al., 1998).

Recent studies indicate that exposure of humans to 1-bromopropane could result in certain neurological disorders (Sclar, 1999; Ichihara et al., 2002). Workers who were exposed to 1-bromopropane through the use of the solvent showed numbness, reduced vibration sensation and superficial touch sensation in the feet, as well as urinary difficulties, diarrhea and/or memory loss. Magnetic resonance images taken for one worker showed increased T_2 weighted signal intensity in the periventricular white matter and the neural area close to the spinal root ganglia. The cases showed various neurological symptoms, but these could not specify the affected site in the CNS. In the present study, the cerebrum was dissected out as a mass composed of the neocortex, hippocampus, piriformcortex, amygdaloid nuclei, caudate putamen, globus pallidus and olfactory tubercle. Therefore, at present, it is difficult to extrapolate our findings in the rat cerebrum with clinical signs reported in humans.

The present study also suggests a possible underlying mechanism of toxicity following long-term exposure to 1-bromopropane. The neurotoxicity of 1-bromopropane includes a reduction in CK levels in the CNS; CK plays an important part in continuous replenishment of ATP from phosphocreatine in neurons, astrocytes, and oligodendrocytes (Wyss et al., 1992). The significant change in the ratio of CK activity/CK-B concentration suggests that the decrease in CK-B amount could not fully explain the decrease in CK activity. 1-Bromopropane might affect CK activity in the rat CNS by both enzymatic inhibition and reduction of the amount of CK.

Decrease in GSH and non-protein-bound SH base in brain regions might be caused by conjugation of 1-bromopropane or its oxidized compounds with glutathione. The proposed main metabolic pathways of 1-bromopropane in rats are direct conjugation with glutathione or conjugation after oxidation at C2 or C3 of 1-bromopropane, although there is also a minor pathway of hydrolysis to 1-propanol (Jones and Walsh, 1979). Glutathione level could be also influenced by glutathione *S*-transferase activity, but the activity of this enzyme was not measured in the present study. In contrast to brain regions, the spinal cord showed increase in GSH. This opposite tendency in the spinal cord seems to be a compensatory phenomenon, but it is difficult to understand this result mechanistically at present. Decrease in GSH and non-protein-bound SH base might be the underlying mechanism of neurotoxicity of 1-bromopropane, since many studies on chemically-induced neurotoxicity suggested the involvement of glutathione depletion with oxidative injury to neuronal or glial cells in rats (Trenge et al., 1991; Shukla et al., 1988). The previous 7-day study showed a decrease in only non-protein-bound SH base and glutathione, but not in protein-bound SH base. However, the present study showed a decrease in protein-bound SH base as well as non-protein-bound SH base. Functional protein containing SH base could also react with 1-bromopropane or its oxidized form. Although no direct evidence was provided in the present study, the reduction in protein-bound SH base suggests modification of functional proteins containing SH base. This might be represented by CK, which contains SH base as the critical site that maintains enzymatic activity (Zhou and Tsou, 1987).

Previous studies reported marked enhancement of Hsp27 expression in cultured cells depleted of glutathione by agents such as diethyl maleate and buthionine sulfoximine (Ito et al., 1998). The increased levels of Hsp27 in the cerebellum, brainstem and spinal cord noted in our study might be related to oxidative stress and glutathione depletion induced by 1-bromopropane.

However, the result should be interpreted cautiously because GSSG, another marker of oxidative stress, increased only in the cerebrum, and there was a discrepancy between the regions that showed increased levels of Hsp27 and GSSG.

Another recent study on 1-bromopropane toxicity did not demonstrate any histological changes in the CNS or peripheral nerves (Sohn et al., 2002). In contrast, our three morphological studies (Yu et al., 1998; Ichihara et al., 2000; Wang et al., 2002) showed clear histological evidence of 1-bromopropane neurotoxicity at 800 and 1000 ppm. However, even in our studies, morphological changes in the CNS were limited to the preterminal axons in gracile nucleus at 800 ppm or more (Ichihara et al., 2000; Wang et al., 2002; Yu et al., 1998), apart from degeneration of Purkinje cells in the cerebellum, which were noted in rats exposed to 1000 ppm (Yu et al., 1998). The present study suggested possible adverse effect of 1-bromopropane on neurons in higher brain regions, as shown by decrease in neuron-specific protein associated with a decrease in the wet weight of the cerebrum.

In conclusion, long-term exposure of rats to 1-bromopropane resulted in a decrease in neuron-specific γ-enolase in the cerebrum, suggesting the involvement of biochemical changes in neurons in the decrease of wet weight of the cerebrum. Glutathione depletion or modification of SH base of functional proteins might underlie the mechanism of 1-bromopropane neurotoxicity. The biochemical changes especially in the cerebrum suggest that long-term exposure to 1-bromopropane might have adverse effects on the CNS.

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