— **Regular Article** —

Toxicity and Oxidative Stress Induced by Organic Arsenical Diphenylarsinic Acid and Inorganic Arsenicals and Their Effects on Spatial Learning Ability in Mice

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In the present study, we investigated the oxidative stress induced by arsenical compounds in mice. Catalase (CAT) activity in the liver was decreased significantly when an organic arsenical compound, diphenylarsinic acid (DPAA), was administered to mice for 7 and 28 consecutive days. Reduced glutathione (GSH) was decreased significantly in the blood of the mice treated for 7 days with another organic arsenical, phenylmethyl arsonic acid (PMAA), and an inorganic arsenical, Na_2HAsO_4, as well as DPAA. GSH was also decreased significantly in the brain of the mice treated for 28 days with DPAA. Arsenic concentrations in the brain of the mice administered DPAA for 7 or 28 days were 1.6–3.0 times higher than those in the livers of these mice. The levels were about 3.0–14 times higher than those in the brains of mice administered inorganic arsenicals, suggesting that organic arsenicals tended to be more highly accumulated in the brain than inorganic arsenicals. The Morris water maze test supported these results, *i.e.*, the latency time of the mice administered DPAA was significantly longer than that of the mice administered Na_2HAsO_4 or $NaAsO_2$, as well as that of controls without arsenical. These results showed that DPAA decreased GSH in the blood, liver and brain, and that DPAA was more easily transferred to the brain. It was suggested that spatial learning ability was depressed by the accumulation of DPAA in the brain.

Key words —— oxidative stress, diphenylarsinic acid, organic arsenical, inorganic arsenical, glutathione, Morris water maze

INTRODUCTION

In 2003, the organic arsenical diphenylarsinic acid (DPAA) was detected in the drinking well water of an apartment building in Kamisu-city, Japan.[1)] The inhabitants who had been drinking the well water exhibited central nervous system disorders,[2,3)] including an ataxic gait, tremors of the extremities, myoclonus, insomnia and memory impairment, which were different from the symptoms of skin pigmentation and peripheral neuropathy observed following chronic exposure to inorganic arsenical.[4,5)]

There have been reports of effects on brain monoamine metabolism in mice after exposure to arsenic trioxide and effects on the central nervous system in rats and guinea pigs after exposure to sodium arsenite.[6,7)] However, since there had been virtually no investigations of the biological effects of DPAA prior to this poisoning case, sufficient knowledge of the toxicity has not yet been obtained. Recently, the role of reactive oxygen species in various diseases has been noted. It has been reported that inorganic arsenicals are one of many chemical substances that can induce reactive oxygen species,[8,9)] and that the oxidative stress causes cell dysfunction when reactive oxygen species are generated above and beyond the antioxidative capacity of the cell. A variety of indicators of oxidative stress have been investigated to date: lipid peroxides (LPO) generated by reactive oxygen species, catalase (CAT) which decomposes hydrogen perox-

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ide, and reduced glutathione (GSH) which protects the cell from the oxidative damage.[10,11] Some investigations reported that sodium arsenite caused significant decreases in GSH and CAT in the liver of mice after intraperitoneal (i.p.) administration of 2.5 mg/kg body weight for three weeks, and in the liver of rats after oral administration at a dose of 10.5 mg/kg body weight.[12,13] Santra *et al.* also reported significant decreases in GSH and CAT in the liver of mice after providing drinking water contaminated with arsenite and arsenate (3.2 mg/l as arsenic) for nine months.[14]

In the present study, we exposed mice to DPAA orally, and investigated the changes of LPO, CAT and GSH levels as indicators of oxidative stress toxicity. It has been reported that the arsenic concentration of the drinking water, which caused health hazard, was 4.5 mg/l, and we decided to use the arsenic concentration 10 mg/l for administration to understand the effect of DPAA more clearly.[1] At the same time, we used mice orally exposed to phenylmethyl arsonic acid (PMAA), as an organic arsenical, and disodium hydrogen arsenate heptahydrate or sodium arsenite, as inorganic arsenicals, to investigate the differences of toxicity between organic and inorganic arsenicals. Although many reports on the health hazard effects of the administration of inorganic arsenicals have been conducted in various experimental designs, for example, in single or continuous administration, and for short or long periods of administration, there are few reports on the effect of the administration of DPAA. Therefore, we investigated in the present study the effect of 7 and 28 days administration of arsenicals in order to obtain as much as possible information, including the difference between the effects based on the exposure period. We considered that DPAA generates reactive oxygen species, and that oxidative stress causes central nervous symptoms, and we examined the effect on the central nervous system of mice by Morris water maze test to measure changes in memory learning and space perception. A method has been established to measure the spatial learning ability of rats and mice using the Morris water maze test.[15–17] We applied this method to the mice in which DPAA or inorganic arsenical was administered. Herein, we report interesting results on the toxicity of the organic arsenical, DPAA, and an inorganic arsenical.

MATERIALS AND METHODS

Animals —— Male ddY mice (3 weeks of age, Japan SLC Inc., Hamamatsu, Japan) were housed under standard laboratory conditions (22 ± 2°C, 60–65% humidity, 12 hr/12 hr light/dark cycle) for a 1-week acclimation period. Mice were offered food pellets (CE-2; Nippon Clea Co., Tokyo, Japan) and water *ad libitum* during the acclimation and experiment periods.

All experiments were performed in accordance with the "Institute of Public Health guide for the care and use of laboratory animals" of the Ibaraki Prefectural Institute of Public Health.

Preparations of Arsenic Solutions —— Thirty five mg of DPAA (Wako Pure Chemical Ind., Ltd., Osaka, Japan), 26.7 mg of PMAA (Hayashi Pure Chemical Ind., Ltd., Osaka, Japan), 41.7 mg of disodium hydrogen arsenate heptahydrate (Wako Pure Chemical Ind., Ltd.), and 17.3 mg of sodium arsenite (Wako Pure Chemical Ind., Ltd.) were dissolved into 1000 ml of purified water (10 mg/l as arsenic), for preparations of DPAA, PMAA, Na_2HAsO_4, and $NaAsO_2$ experimental arsenic solutions, respectively.

7-day Experiment —— Mice were divided into three groups, each containing six mice. Mice in the control group 7-Cont(1) were administered distilled water as drinking water. Group 7-DPAA was administered the DPAA solution, and group 7-Na_2HAsO_4 was administered the Na_2HAsO_4 solution in the drinking water. For separate 7-day experiment, additional mice were divided into three groups, those were, 7-Cont(2), 7-PMAA, and 7-$NaAsO_2$ groups, each containing six mice. Mice in the control group 7-Cont(2) were administered distilled water as drinking water. Group 7-PMAA was administered the PMAA solution, and group 7-$NaAsO_2$ was administered the $NaAsO_2$ solution in the drinking water.

28-day Experiment —— Mice were divided into two groups, each containing six mice. Mice in the control group 28-Cont were administered distilled water as drinking water. Group 28-DPAA was administered the DPAA solution. Food pellets and water were provided to all groups *ad libitum* during the 7-day and 28-day experimental protocols. During administration, body weight of the mice and intake of the water were measured.

Animals were decapitated after 7 and 28 days exposure in the 7-day and 28-day experiments, respectively. Blood samples were collected in hep-

arinized tubes from the caudal vena cava, and then centrifuged to separate plasma. The erythrocytes were washed in cold isotonic saline to isolate the erythrocyte sample. Samples of the liver and brain were removed after taking blood, and were stored along with the erythrocytes at −80°C until use.

Biochemical Assay —— LPO in plasma was determined by the method of Yagi.[18] The plasma was precipitated using N/12 sulfuric acid and 10% phosphotungstic acid, and reacted with thiobarbituric acid (TBA) at 95°C for 60 min. After cooling, *n*-butanol was added to the mixture and shaken. The mixture was then centrifuged at 1500 *g* for 10 min, and the butanol phase obtained was measured at 515 nm excitation and 553 nm emission by a spectrofluorometer (Hitachi F-4010). The standard for LPO, 1,1,3,3-tetraethoxypropane (Wako Pure Chemical Ind., Ltd.), was made to react with the TBA reagent, and the reaction product was measured similarly. The concentration of LPO in plasma was expressed as nanomoles of malondialdehyde equivalents (nmol MDA/ml).

LPO in the liver and the brain was determined by the method of Uchiyama *et al.*[19] The liver or the brain sample was homogenized in nine volumes (w:v) of 1.15% potassium chloride. The homogenate was mixed with 1% H_3PO_4 and 0.67% TBA and then heated at 95°C for 45 min. After cooling, *n*-butanol was added to the mixture and shaken, and the mixture was then centrifuged at 1500 *g* for 10 min. The reaction product in the butanol phase was measured at 535 nm and 520 nm by spectrophotometer (Hitachi U-4310). The difference in the optical densities between the two wavelengths (A535–520) was calculated as the LPO. The standard for LPO, 1,1,3,3-tetraethoxypropane, was reacted with the TBA reagent, and the reaction product was measured similarly. The concentrations of LPO in the liver and brain were expressed as nanomoles of malondialdehyde equivalents per gram tissue (nmol MDA/g tissue).

CAT activity was measured by the method of Aebi.[20] After the addition of four volumes of purified water, the erythrocyte sample became the hemolysate sample. The liver or brain samples were homogenized in nine volumes (w:v) of 0.25 M sucrose solution, and centrifuged at 700 *g* for 10 min. The supernatant was then centrifuged at 7000 *g* for 10 min, and the precipitate, as the crude mitochondrial fraction obtained after centrifugation, was utilized as the tissue sample. The hemolysate or the tissue samples were diluted in 50 mM phosphate buffer (pH 7.0). The reaction was initiated by adding 1.0 ml of 30 mM H_2O_2 as the substrate to 2.0 ml of the diluted sample solution. The decrease in H_2O_2 concentration of the reaction mixture was recorded continuously at 240 nm by spectrophotometer, against a blank containing 1.0 ml of 50 mM phosphate buffer instead of substrate. Activity was calculated by comparing the absorbance with a standard curve generated from known catalase concentrations (Catalase, from bovine liver, 12000 units/mg, Wako Pure Chemical Ind., Ltd.).

The GSH levels in blood were measured by the method of Miwa *et al.*[21] Erythrocytes were added to distilled water and metaphosphoric acid solution (100 ml containing 1.67 g glacial metaphosphoric acid, 0.2 g disodium EDTA, and 30 g sodium chloride) and mixed. The mixture was centrifuged at 8000 *g* for 10 min. The GSH fraction supernatants were added to 0.3 M Na_2HPO_4 and 5,5′-dithiobisnitrobenzoic acid (DTNB). The absorbance of the yellow substance, which was obtained in this reaction, was measured at 412 nm by spectrophotometer for the estimation of GSH.

GSH in the liver and brain was measured using a GSH/GSSG-412 kit (Bioxytech) at 412 nm with a spectrophotometer. The liver or brain samples were used for the test, after homogenization in nine volumes (w:v) of 0.25 M sucrose solution.

Arsenic Concentration —— Arsenic concentrations in the liver or brain were measured by the method of Ishizaki.[22] Liver or brain samples (0.1–0.2 g) were cut into smaller and equal sizes, and placed in a china crucible. Samples were soaked in 2 ml of 50% magnesium nitrate solution, and incinerated at 550°C for 6 hr after drying at 50°C for 12 hr. After cooling, the mixture was added to 5 ml of 10 N HCl, and then heated to 50°C for 30 min. The solution was transferred to a separating funnel, and 10 N HCl solution, in which the crucible was washed, was added to a separating funnel. Moreover, 1 ml of 40% potassium iodide (KI) solution was added to the separating funnel and mixed. The mixture was left for 3 min to reduce pentavalent arsenic to trivalent arsenic. The trivalent arsenic produced in the mixture was extracted 2 times in 5 ml of chloroform, and the chloroform fraction was extracted in 2 ml of 0.025% magnesium nitrate solution. The arsenic in the sample solution was measured by atomic absorption spectrophotometer.

Morris Water Maze Test —— The water maze experiment was based on the method of Morris and Troen.[15, 16] In this experiment, additional ddY mice

were used along with the mice for the 7-day and 28-day experiments. Male ddY mice (3 weeks of age) were housed under standard laboratory conditions for a 1-week acclimation period and 1 week of training. Mice were provided with food pellets (CE-2) and water *ad libitum* during the acclimation and experimental periods. Mice were divided into four groups, each containing 6–7 mice. Mice in the control group Morris Water Maze (MWM)-Cont were administered distilled water as drinking water. The MWM-DPAA group was treated with the DPAA solution, the MWM-Na_2HAsO_4 group was treated with the Na_2HAsO_4 solution, and the MWM-$NaAsO_2$ group was treated with the $NaAsO_2$ solution in the drinking water. The concentration of each arsenical in the drinking water of each treatment group was 10 mg/l as arsenic. A pool of 90 cm × 45 cm (diameter × height, Nakayama Co. Ltd., Hitachi, Japan) was used for the experiment. In approaching the center from the inner wall of the pool, the escape platform, 10 cm in diameter, was placed in one of the pool quadrants. In adding the skim milk to the pool, the water was filled to a height of 1 cm from the surface of the platform. The water temperature was set at about 25°C. The mouse was allowed to swim from an arbitrary place along the poolside. The latency time until the mouse reached the platform below the water surface was measured. The tester guided or carried the mouse to the platform when the latency time was over 90 sec. The test was conducted in three trials each day, and the mean time expressed as the mean latency time of the mouse for the experiment day. The test was carried out every day from the administration up to 2 weeks, and then one day per week up to 9 weeks from the administration. Shelves and tables were placed in the same position as a landmark around the pool during the test.

Statistical Analysis —— Data for the 7-day and 28-day administration experiments were analyzed using Student's *t*-test. In the Morris water maze experiment, data between each administered group from the onset to 9 weeks were analyzed by a two-way analysis of variance (ANOVA), followed by Tukey-Kramer's *post hoc* tests, and the data between each administration group for each week were analyzed using Student's *t*-test. Values of 5% or less ($p < 0.05$) were considered significant. The relationship between arsenic content, LPO, CAT and GSH was investigated by determining Pearson's correlation coefficient.

RESULTS

Body Weight and Intake of Drinking Water

No significant change in the body weight and intake of drinking water was observed between the groups treated with arsenicals in the 7-day experiment. The body weight of the mice in group 28-DPAA temporarily declined after 7 days of administration compared with the group 28-Cont, and recovered gradually by 28 days of administration. Intake of drinking water in group 28-DPAA decreased and recovered, similarly to changes in the body weight. There was no characteristic difference in general motor activity between the treated and control groups in both the 7- and 28-day experiments.

Changes of LPO and CAT

The intake of substantial arsenic of the mouse was 2.47 mg As/kg per day (total quantity: 17.28 mg/kg) and 1.76 mg As/kg per day (total quantity: 49.23 mg/kg) in the 7-DPAA and 28-DPAA groups, respectively. This arsenic quantity was calculated based on the drinking water intake of the mouse.

The results of the LPO and CAT analysis are shown in Table 1. No significant changes in LPO were observed in the blood, liver or brain in group 28-DPAA compared with the group 28-Cont. The concentrations of LPO in the blood, liver and brain were virtually the same between group 7-DPAA and 7-Cont(1). No significant change in CAT in blood was observed in group 7-DPAA or 28-DPAA compared with group 7-Cont(1) or group 28-Cont, respectively. A significant change in CAT in the liver was only observed in groups 7-DPAA and 28-DPAA compared with groups 7-Cont(1) and 28-Cont, respectively ($p < 0.001$, $p < 0.01$). CAT activities were below the detection limit in the brains for all groups.

Changes of GSH

The arsenic intake was 2.47 mg As/kg per day (total quantity: 17.28 mg/kg) in group 7-DPAA, 2.15 mg As/kg per day (total quantity: 15.05 mg/kg) in group 7-PMAA, 2.65 mg As/kg per day (total quantity: 18.57 mg/kg) in group 7-Na_2HAsO_4 and 2.22 mg As/kg per day (total quantity: 15.54 mg/kg) in group 7-$NaAsO_2$, respectively. The intake in group 28-DPAA was 1.76 mg As/kg per day (total quantity: 49.23 mg/kg). There were no differences in intake between organic arsenic and inorganic arsenic.

Table 1. Changes in LPO and CAT in the Blood, Livers and Brains of Mice after DPAA Exposure

Treatment group	LPO			CAT		
	plasma (nmol MDA/ml)	liver (nmol MDA/g tissue)	brain	erythrocyte (k units/ml)	liver (k units/g tissue)	brain
7-Con(1)	26.8 ± 6.4	172 ± 51	134 ± 57	19.4 ± 3.1	16.9 ± 2.4	—
7-DPAA	32.4 ± 8.1	171 ± 32	157 ± 23	20.6 ± 3.3	8.9 ± 3.0***	—
28-Cont	38.5 ± 19.6	261 ± 34	182 ± 46	16.1 ± 2.7	19.5 ± 4.0	—
28-DPAA	38.2 ± 15.0	210 ± 87	208 ± 33	13.2 ± 2.9	12.2 ± 3.4**	—

Values are expressed as the mean ± S.D. of 6 mice. ** $p < 0.01$, *** $p < 0.001$, compared with the control group. —: below the detection limit.

Table 2. Changes in GSH in Erythrocytes, Livers and Brains of Mice after Organic or Inorganic Arsenical Exposure

Treatment group		erythrocyte [mg/dl red blood cell (RBC)]	liver (μmol/g tissue)	brain
7-Cont(1)		22.4 ± 3.9	11.00 ± 0.72	1.49 ± 0.42
7-DPAA	organic	14.8 ± 3.4**	8.75 ± 0.72***	1.56 ± 0.09
7-Na_2HAsO_4	inorganic	22.6 ± 3.4	10.06 ± 0.61*	1.86 ± 0.29
7-Cont(2)		21.8 ± 5.8	7.93 ± 1.61	2.35 ± 0.24
7-PMAA	organic	20.5 ± 2.0	5.92 ± 1.19*	2.41 ± 0.19
7-$NaAsO_2$	inorganic	23.7 ± 3.9	6.81 ± 1.56	2.34 ± 0.12
28-Cont		22.4 ± 6.9	9.31 ± 1.15	1.99 ± 0.27
28-DPAA	organic	17.6 ± 5.3	8.78 ± 0.70	1.66 ± 0.26*

Values are expressed as the mean ± S.D. of 6 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

The results of the GSH analysis are shown in Table 2. GSH concentrations in the erythrocytes and livers of group 7-DPAA decreased significantly compared with those of group 7-Cont(1) ($p < 0.01$, $p < 0.001$, respectively); however, in the brain, the values were almost the same as that of group 7-Cont(1). The GSH concentrations of group 7-Na_2HAsO_4 decreased significantly in the liver compared with group 7-Cont(1) ($p < 0.05$); in contrast, the values in the erythrocytes and brain were almost same as those of group 7-Cont(1). In group 7-PMAA, which was treated with another organic arsenical, PMAA, GSH concentrations in the liver decreased significantly compared with those of group 7-Cont(2) ($p < 0.05$), although the levels in the erythrocytes and brain were almost same as those in the 7-Cont(2) group. In group 7-$NaAsO_2$, which was treated with inorganic arsenical $NaAsO_2$, the GSH concentrations in the erythrocytes, liver and brain were almost the same as those of group 7-Cont(2). In group 28-DPAA, which was treated with DPAA for 28 days, the GSH concentrations in the erythrocytes and liver were almost the same as those of group 28-Cont; however, the concentrations decreased in group 7-DPAA, which was treated with DPAA for 7 days. On the other hand, GSH concentrations in the brain decreased significantly compared with group 28-Cont ($p < 0.05$).

Arsenic Concentrations in the Liver and the Brain after Administration of Inorganic and Organic Arsenicals

The results of arsenic determination are shown in Table 3. In the brain, the arsenic concentration (0.921 ± 0.178 μg/g tissue) of group 7-DPAA, which was treated with organic arsenic DPAA for 7 days, was about three times higher than that in the liver (0.333 ± 0.105 μg/g tissue). The arsenic concentration in the brains of group 7-Na_2HAsO_4, which was treated with inorganic arsenical Na_2HAsO_4, was 0.065 ± 0.044 μg/g tissue. In the experiment using additional mice, arsenic concentrations in the brains of group 7-DPAA, which were treated with organic arsenical DPAA for 7 days, were 0.170 ± 0.074 μg/g tissue and about three times higher than that in the brain of group 7-$NaAsO_2$ (0.052 ± 0.032 μg/g tissue). Moreover, arsenic concentrations in the brain (0.590 ± 0.194 μg/g tissue) of group 28-DPAA, which was treated with organic arsenic DPAA for 28 days, were about 1.6 times higher than those in the liver (0.374 ± 0.124 μg/g tissue).

Morris Water Maze Test

In the Morris water maze test results (Fig. 1), the mean latency time of group MWM-Cont to the platform was 25 sec on the first day, which decreased

Table 3. Arsenic Concentration in the Livers and Brains of Mice after Organic or Inorganic Arsenical Exposure

Treatment group		liver	brain
		(μg/g tissue)	
7-Cont(1)		0.020 ± 0.009	0.020 ± 0.019
7-DPAA	organic	0.333 ± 0.105***	0.921 ± 0.178***
7-Na_2HAsO_4	inorganic	NT	0.065 ± 0.044*
7-Cont(2)		NT	0.015 ± 0.009
7-PMAA	organic	NT	0.170 ± 0.074**
7-$NaAsO_2$	inorganic	NT	0.052 ± 0.032*
28-Cont		0.015 ± 0.011	0.015 ± 0.011
28-DPAA	organic	0.374 ± 0.124***	0.590 ± 0.194***

Values are expressed as the mean ± S.D. of 6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control group. NT: not tested.

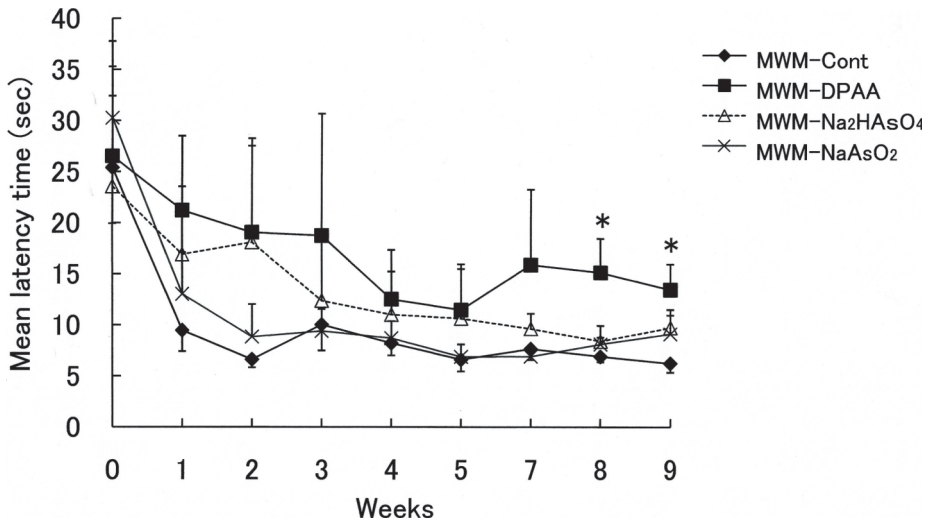

Fig. 1. Changes in the Mean Latency Time for the Morris Water Maze Test in Mice after Organic or Inorganic Arsenical Exposure
Mice were trained in 3 trials per day. Values are expressed as the mean ± S.E. of 6–7 mice. $^{*}p < 0.05$, compared with the control group.

rapidly during the learning for 1–2 weeks, and by 5–10 sec after 3 weeks, showing constancy or decline. Differences in the mean latency time of individual mice were small and stabilized comparatively. On the other hand, the mean latency time of group MWM-DPAA decreased slightly, increased transiently during after 7 weeks learning, and decreased slightly again thereafter. The latency time of the MWM-DPAA group was significantly increased, longer than that of the MWM-Cont group during 1–4 weeks of the experiment in ANOVA ($F_{(1,57)} = 4.47$; $p < 0.05$). In addition, a significant change was observed between the MWM-Cont and MWM-DPAA groups during the experiment for 0–9 weeks ($F_{(1,126)} = 8.38$; $p < 0.01$). The mean latency time of group MWM-Na_2HAsO_4, which decreased slightly during the learning for 4 weeks, was about 10 sec during the 5 weeks after learning; however, this decrease was not less than that of group MWM-DPAA. In group MWM-$NaAsO_2$, the mean latency time was almost the same as that of group MWM-Cont; it was slightly higher than that of the other groups on the first day (30 sec). However, a significant change was observed between the MWM-DPAA and MWM-$NaAsO_2$ groups during the learning for 0–9 weeks ($F_{(1,126)} = 5.55$; $p < 0.05$). In each group at 8 weeks, the mean latency time of group MWM-DPAA was significantly higher than that of group MWM-Cont (15.13 ± 3.32 sec, 6.91 ± 0.54 sec, respectively, $F_{(1,12)} = 5.08$; $p < 0.05$). Moreover, in each group at 9 weeks, the mean latency time of group MWM-DPAA was significantly higher than that of group MWM-Cont (13.44 ± 2.51 sec, 6.24 ± 0.88 sec, respectively, $F_{(1,13)} = 7.34$; $p < 0.05$).

DISCUSSION

Maiti *et al.* reported a significant decrease in GSH and an increase in LPO in the liver of rats i.p. administered sodium arsenic at its LD_{50} dose (15.86 mg/kg).[23] Rabbani *et al.* also reported a significant decrease in GSH and an increase in LPO in the blood of rabbits after administration of arsenic trioxide at 3–5 mg/kg per day for 7 days.[24] In the present study, there was no significant change in LPO in the blood, liver, or brain (Table 1). This may be due to differences in animal species and strains of mice.

Bashir *et al.*[13] and Santra *et al.*[14] reported that decreases in GSH and CAT were observed in the liver of mice or rats after the administration of arsenical. Thus, inorganic arsenic decreased the CAT activity of mice in the liver. In this study, a significant decrease in CAT activity in the liver was also observed in the mice administered organic arsenical DPAA for 7 and 28 days (Table 1). CAT catalyzes hydrogen peroxide into water, and prevents cellular damage by hydrogen peroxide. Based on the different processes, GSH and the glutathione oxidation-reduction cycle likely play a role that is important for the prevention of cellular damage. For lipid, protein and carbohydrate, active oxygen and free radicals generated by various triggers cause oxidation, protein degeneration, inactivation of the enzyme, etc., thus, they consequently injure the cell. Oshino *et al.* showed the complementary effect of CAT and glutathione peroxidase (GSH-Px) when they determined the peroxidation prevention of hydrogen peroxide and lipid.[25] About 50–60% of hydrogen peroxide was decomposed by CAT when hydrogen peroxide was injected at doses less than 2 μmol/min per g tissue to the liver of rats, and the remaining hydrogen peroxide was decomposed by GSH-Px. Through GSH-Px, hydrogen peroxide and peroxidized organic compounds are removed, and GSH is converted into oxidized glutathione (GSSG). The decrease in GSH means a lowering of the defense function against oxidative changes in cells. A significant decrease in GSH was also observed in erythrocytes and livers in this study, suggesting that GSH in the blood and liver was decreased as a result of protection against oxidative changes in the cells, and we observed that CAT in the liver was also similarly decreased. It has been reported that inorganic arsenic decreases GSH in the blood and liver.[26, 27]

Exposure to organic arsenical DPAA for 7 days caused a significant decrease in GSH, but the levels recovered after exposure for 28 days, as shown as Table 2. Kuhs *et al.* reported that lethal doses of some arsenical derivatives of benzene, such as Stovarsol, Carbarsone, Tryparsamide, Proparsanol, and Mepharsen, and those derivatives of naphthalene and quinine, tended to be increased in rats, but no such increase in the lethal dose, or so-called "tolerance," was observed in sodium cacodylate or sodium arsenite.[28] Although the existence and mechanism of the tolerance in arsenic administration has not yet been fully elucidated, tolerance to organic arsenicals may appear more readily than tolerance to inorganic arsenicals.

The metabolism of DPAA has been examined in a toxicity test report.[29] In rats, which were administered a single dose of ^{14}C-DPAA (0.3 mg/kg), the concentration of DPAA in most tissues exhibited the highest values 4 hr after administration, and was more than 3% of the respective dose. Subsequently, the concentration of DPAA diminished in a time-dependent manner, to a level less than 10% of the highest value or below the detection limit in the blood and liver 168 hr after administration. On the other hand, the highest concentrations of DPAA in the cerebrum and the cerebellum were observed 24 hr after administration, and were only 0.102% and 0.020% of the dose, respectively. However, 168 hr after the administration, that value remained at 20% of the highest value. It would appear that the storage and metabolism of the organic arsenical DPAA in the brain differ from that in other organs. Ishizaki reported that the accumulation of arsenic was observed in the brain when the inorganic arsenical sodium arsenite was administered to rats chronically and continuously, although the transfer of arsenic to the brain was less than that to other organs.[30] As shown in Table 3, the concentration in the brain after 7 days of administration of organic arsenical DPAA was 0.921 ± 0.178 μg/g tissue, about 3 times higher than that in the liver (0.333 ± 0.105 μg/g tissue). The arsenic concentration in the brain after 7 days of administration of the inorganic arsenical, Na_2HAsO_4, was only 0.065 ± 0.044 μg/g tissue. Similarly, the arsenic concentration in the brain after 7 days of administration of organic arsenical PMAA was 0.170 ± 0.074 μg/g tissue, and was about 3 times higher than that after 7 days administration of inorganic arsenical $NaAsO_2$ (0.052 ± 0.032 μg/g tissue). Further, the concentration of arsenic in the brain

(0.590 ± 0.194 μg/g tissue) after 28 days of administration of DPAA was also about 1.6 times higher than that in the liver (0.374 ± 0.124 μg/g tissue). It was suggested that the organic arsenical was easily transferred to the brain, and accumulated more readily in the brain compared with inorganic arsenical, and also that DPAA tended to accumulate in the brain more easily than PMAA (Table 3). Such transfer of the organic arsenical to the brain is supported by the fact that organic arsenical has higher lipophilicity than inorganic arsenical. It has been reported that the partition coefficient (log Pow) of DPAA is 1.2 at pH 3.[29)]

Previously, Kannan *et al.* reported that significant decreases in GSH and dopamine were observed in the brain when rats were administered drinking water containing 25 mg/l arsenic as sodium arsenite for 16 weeks.[7)] Miyagawa *et al.* analyzed the behavior and investigated the influence on the central nervous system in mice treated with DPAA.[31)] They reported that mice treated with 1 or 5 mg of DPAA/kg, once a day for 10 days significantly decreased the time until the mouse fell down from the rod in the Rota-rod test, compared with the non-treated control mouse. In addition, they revealed that the impairment of motor coordination by DPAA depended on the hypoactivity of dopamine receptors in the striatum. Brain function related to space perception appears to be a network consisting of various central neurons, such as the hippocampus, striatum, basal nucleus, cerebral neocortex and cerebellum. It has been reported that mice administered drinking water containing 30 mg of DPAA/l (8.6 mg/l as arsenic) or 100 mg/l (28.6 mg/l as arsenic), showed significantly higher ambulatory activity 2 weeks after administration compared with non-treated mice, and continued the level by 24 weeks, suggesting that DPAA caused an excitatory effect on central neurons due to the analeptic effect by which the motor activity of the mouse increased.[32)]

As shown in Fig. 1, the latency time of the MWM-DPAA group was significantly prolonged in comparison with the MWM-Cont group during learning for 1–4 weeks in ANOVA ($p < 0.05$). In addition, the latency time of the DPAA group also increased significantly at 8 weeks compared with the control group in Student's *t*-test ($p < 0.05$). This suggested that DPAA more easily reduced the ability of space perception by affecting the central neurons. These results can help to explain why DPAA was more easily transferred to the brain, as measured by the brain arsenic content after 7 days of administration, unlike the inorganic arsenical.

The symptoms of DPAA poisoning in Kamisucity were mainly central nervous system symptoms, which is consistent with the symptoms of Minamata disease caused by methyl mercury compounds. From the above fact, it was found that DPAA decreased GSH in the blood, liver and brain, and that DPAA was more easily transferred to the brain compared with other arsenicals. Thus, it was suggested that spatial learning ability was easily depressed as a central nervous system symptom.

REFERENCES

1) Ishizaki, M., Yanaoka, T., Nakamura, M., Hakuta, T., Ueno, S., Komuro, M., Shibata, M., Kitamura, T., Honda, A., Doi, M., Ishii, K., Tamaoka, A., Shimojo, N., Ogata, T., Nagasawa, E. and Hanaoka, S. (2005) Detection of bis (diphenylarsine) oxide, diphenylarsinic acid and phenylarsonic acid, compounds probably derived from chemical warfare agents, in drinking well water. *J. Health Sci.*, **51**, 130–137.
2) Ishii, K., Tamaoka, A., Iwasaki, N. and Otsuka, F. (2004) Intoxication of organic arsine in Kamisu, Japan. *Jpn. J. Toxicol.*, **17**, 125–131 (in Japanese).
3) Ishii, K., Tamaoka, A., Otsuka, F., Iwasaki, N., Shin, K., Matsui, A., Endo, G., Kumagai, Y., Ishii, T., Shoji, S., Ogata, T., Ishizaki, M., Doi, M. and Shimojo, N. (2004) Diphenylarsinic acid poisoning from chemical weapons in Kamisu, Japan. *Ann. Neurol.*, **56**, 741–745.
4) Rahman, M. M., Chowdhury, U. K., Mukherjee, S. C., Mondal, B. K., Paul, K., Lodh, D., Biswas, B. K., Chanda, C. R., Basu, G. K., Saha, K. C., Roy, S., Das, R., Palit, S. K., Quamruzzaman, Q. and Chakraborti, D. (2001) Chronic arsenic toxicity in Bangladesh and West Bengal, India-A Review and Commentary. *J. Toxicol. Clin. Toxicol.*, **39**, 683–700.
5) Yamauchi, H., Yoshida, T., Aikawa, H., Aminaka, M., Nito, Y. and Yoshida, K. (2000) Study on the metabolism of arsenic in the patients with chronic arsenic poisoning. *Jpn. J. Clin. Ecol.*, **9**, 68–74 (in Japanese).
6) Itoh, T., Zhang, Y. F., Murai, S., Saito, H., Nagahama, H., Miyate, H., Saito, Y. and Abe, E. (1990) The effect of arsenic trioxide on brain monoamine metabolism and locomotor activity of mice. *Toxicol. Lett.*, **54**, 345–353.
7) Kannan, G. M., Tripathi, N., Dube, S. N., Gupta, M. and Flora, S. J. (2001) Toxic effects of arsenic (III)

on some hematopoietic and central nervous system variables in rats and guinea pigs. *J. Toxicol. Clin. Toxicol.*, **39**, 675–682.
8) Pi, J., Yamauchi, H., Kumagai, Y., Sun, G., Yoshida, T., Aikawa, H., Hopenhayn-Rich, C. and Shimojo, N. (2002) Evidence for induction of oxidative stress caused by chronic exposure of Chinese residents to arsenic contained in drinking water. *Environ. Health Perspect.*, **110**, 331–336.
9) Tabacova, S. and Hunter, E. S. (1993) Modulation of arsenic teratogenicity by free radical scavengers. *Teratology Soc. Abst.*, **47**, 390–391.
10) Konings, A. W. T. and Drijver, E. B. (1979) Radiation effects on membranes. I. Vitamin E deficiency and lipid peroxidation. *Radiat. Res.*, **80**, 494–501.
11) Gultekin, F., Delibas, N., Yasar, S. and Kilinc, I. (2001) In vivo changes in antioxidant systems and protective role of melatonin and combination of vitamin C and vitamin E on oxidative damage in erythrocytes induced by chlorpyrifos-ethyl in rats. *Arch. Toxicol.*, **75**, 88–96.
12) Gupta, R. and Flora, S. J. (2006) Protective effects of fruit extracts of *Hippophae rhamnoides* L. against arsenic toxicity in Swiss albino mice. *Hum. Exp. Toxicol.*, **25**, 285–295.
13) Bashir, S., Sharma, Y., Irshad, M., Gupta, S. D. and Dogra, T. D. (2006) Arsenic-induced cell death in liver and brain of experimental rats. *Basic Clin. Pharmacol. Toxicol.*, **98**, 38–43.
14) Santra, A., Maiti, A., Das, S., Lahiri, S., Charkaborty, S. K. and Mazumder, D. N. (2000) Hepatic damage caused by chronic arsenic toxicity in experimental animals. *J. Toxicol. Clin. Toxicol.*, **38**, 395–405.
15) Morris, R. G. (1981) Spatial localization does not require the presence of local cues. *Learn. Motiv.*, **12**, 239–260.
16) Troen, A. M., Shea-Budgell, M., Shukitt-Hale, B., Smith, D. E., Selhub, J. and Rosenberg, I. H. (2008) B-vitamin deficiency causes hyperhomocysteinemia and vascular cognitive impairment in mice. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 12474–12479.
17) Matilla, A., Roberson, E. D., Banfi, S., Morales J., Armstrong, D. L., Burright, E. N., Orr, H. T., Sweatt, J. D., Zoghbi, H. Y. and Matzuk, M. M. (1998) Mice lacking ataxin-1 display learning deficits and decreased hippocampal paired-pulse facilitation. *J. Neurosci.*, **18**, 5508–5516.
18) Yagi, K. (1976) A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.*, **15**, 212–216.
19) Uchiyama, M. and Mihara, M. (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.*, **86**, 271–278.
20) Aebi, H. (1984) Catalase *in vitro*. *Methods Enzymol.*, **105**, 121–126.
21) Miwa, S., Luzzatto, L., Rosa, R., Paglia, D. E., Schröter, W., Flora, A. D., Fuji, H., Board, P. G. and Beutler, E. (1989) Recommended methods for an additional red cell enzyme (pyrimidine 5′-nucleotidase) assay and the determination of red cell adenosine-5′-triphosphate, 2,3-diphosphoglycerate and reduced glutathione. *Clin. Lab. Haematol.*, **11**, 131–138.
22) Ishizaki, M. (1977) Determination of total arsenic in biological samples by flameless atomic absorption spectrometry using a carbon tube atomizer. *Bunseki Kagaku*, **26**, 667–672 (in Japanese).
23) Maiti, S. and Chatterjee, A. K. (2001) Effects on levels of glutathione and some related enzymes in tissues after an acute arsenic exposure in rats and their relationship to dietary protein deficiency. *Arch. Toxicol.*, **75**, 531–537.
24) Rabbani, G. H., Saha, S. K., Akhtar, M., Marni, F., Mitra, A. K., Ahmed, S., Alauddin, M., Bhattacharjee, M., Sultana, S. and Chowdhury, A. K. (2003) Antioxidants in detoxification of arsenic-induced oxidative injury in rabbits: preliminary results. *J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng.*, **38**, 273–287.
25) Oshino, N. and Chance, B. (1977) Properties of glutathione release observed during reduction of organic hydroperoxide, demethylation of aminopyrine and oxidation of some substances in perfused rat liver, and their implications for the physiological function of catalase. *Biochem. J.*, **162**, 509–525.
26) Nikaido, M., Pi, J., Kumagai, Y., Yamauchi, H., Taguchi, K., Horiguchi, S., Sun, Y., Sun, G. and Shimojo, N. (2003) Decreased enzyme activity of hepatic thioredoxin reductase and glutathione reductase in rabbits by prolonged exposure to inorganic arsenate. *Environ. Toxicol.*, **18**, 306–311.
27) Rabbani, G. H., Saha, S. K., Akhtar, M., Marni, F., Mitra, A. K., Ahmed, S., Alauddin, M., Bhattacharjee, M., Sultana, S. and Chowdhury, A. K. (2003) Antioxidants in detoxification of arsenic-induced oxidative injury in rabbits: preliminary results. *J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng.*, **38**, 273–287.
28) Kuhs, M. L., Longley, B. J. and Tatum, A. L. (1939) Development of tolerance to organic arsenicals in laboratory animals. *J. Pharmacol. Exp. Ther.*, **66**, 312–317.
29) Ministry of the Environment (2006) *The toxicity testing report of diphenylarsinic acid (DPAA)*, http://www.env.go.jp/chemi/report/h18-08/, pp. 1–

63 (cited 11 May, 2007, in Japanese).
30) Ishizaki, M. (1980) Studies on Pharmacokinetics of Arsenicals in Rats. *Jpn. J. Hyg.*, **35**, 584–596 (in Japanese).
31) Miyagawa, K., Narita, M., Miyatake, M., Kato, K., Yamanaka, K. and Suzuki, T. (2007) Behavioral analysis of chronic exposure to diphenylarsinic acid associated with influence on the central nervous systems. *Nihon Shinkei Seishin Yakurigaku Zasshi*, **27**, 181–189 (in Japanese).
32) Japan Science Foundation (2005) Research on the health effects of diphenylarsinic acid in the 2004 fiscal year, *Research report*, Environmental Policy Bureau, Ministry of the Environment, pp. 69–78 (in Japanese).