FULL PAPER *Public Health*

Detection of *in vivo* DNA Damage Induced by Ethanol in Multiple Organs of Pregnant Mice Using the Alkaline Single Cell Gel Electrophoresis (Comet) Assay

Ryoko KIDO[1,2,3], Itaru SATO[1,2] and Shuji TSUDA[1,2]*

[1] *Department of Applied Veterinary Science, The Doctoral Course of the United Graduate School of Veterinary Science, Gifu University, 1–1 Yanagito, Gifu, Gifu 501–1112,* [2] *Laboratory of Veterinary Public Health, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, 3–18–8 Ueda Morioka, Iwate 020–8550, and* [3] *Biosafety Research Center, Foods, Drugs and Pesticides, 582–2 Shioshinden, Iwata, Shizuoka 437–1213, Japan*

(Received 24 June 2005/Accepted 16 September 2005)

ABSTRACT. Ethanol is principal ingredient of alcohol beverage, but considered as human carcinogen, and has neurotoxicity. Alcohol consumption during pregnancy often causes fetal alcohol syndrome. The DNA damage is one of the important factors in carcinogenicity or teratogenicity. To detect the DNA damage induced by ethanol, we used an *in vivo* alkaline single cell gel electrophoresis (Comet) assay in pregnant mice organs and embryos. Pregnant ICR mice on Day 7 of gestation were treated with 2, 4 or 8 g/kg ethanol, and maternal organs/tissues and embryos were subjected to the Comet assay at 4, 8, 12 and 24 hr after ethanol treatment. Four and 8 g/kg ethanol induced DNA damage in brain, lung and embryos at 4 or 8 hr after the treatment. Two g/kg ethanol did not cause any DNA damage, and 8 g/kg ethanol only increased the duration of DNA damage without distinct increase in the degree of the damage. No significant DNA damage was observed in the liver. To detect the effect of acetaldehyde, disulfiram, acetaldehyde dehydrogenase inhibitor, was administered before 4 g/kg ethanol treatment. No significant increase of DNA damage was observed in the disulfiram pre-treated group. These data indicate that ethanol induces DNA damage, which might be related to ethanol toxicity. Since pre-treatment of disulfiram did not increase DNA damage, DNA damage observed in this study might not be the effect of acetaldehyde.
KEY WORDS: Comet assay, DNA damage, ethanol, pregnant mouse.

J. Vet. Med. Sci. 68(1): 41–47, 2006

Ethanol is the principal ingredient of alcohol beverages. Epidemiological studies indicate that addictive intake of ethanol is related to the cancers in the upper gastrointestinal tract such as oral cavity, pharynx, larynx and esophagus, the large consumption of ethanol increases the incidents of hepatocirrhosis or hepatoma [5, 7]. Ethanol is classified into Group 1 (human carcinogen) by International Agency for Research on Cancer (IARC) [7]. Ethanol has also neurotoxicity, and the nerve system is easily affected by ethanol. The cortical atrophy or alcoholic cerebellar atrophy are often reported in chronic alcoholic patients [6]. The large consumption of ethanol in the pregnancy causes fetal alcohol syndrome (FAS), which is characterized by growth retardation, facial anomalies such as short palpebral fissures, flat mid face, maxillary hypoplasia, and mental retardation of the newborns [2, 7, 10, 21].

There are many *in vivo* or *in vitro* studies about the toxicity of ethanol, especially its neurotoxicity [6]. But the mechanism of ethanol toxicity, such as carcinogenicity or teratogenicity is not clear. It is generally considered that DNA damage play an important role in the carcinogenesis or congenital anomalies [5]. There was a report showing that significant increase of DNA damage was observed in rat brain administered ethanol [23]. Thus, DNA damage could be a reason for the neurotoxicity, carcinogenicity or congenital anomalies induced by ethanol. Acetaldehyde, a metabolite of ethanol, could play an important role in ethanol toxicity by crosslinking the DNA [8, 26].

To detect the DNA damage induced by the ethanol, we applied the *in vivo* alkaline single cell gel electrophoresis (Comet) assay [22–24] to multiple organs in pregnant mice. Furthermore, we administered disulfiram (DSF), the acetaldehyde dehydrogenase inhibitor, to pregnant mice so as to investigate the effects of acetaldehyde [9, 11, 19].

* CORRESPONDENCE TO: TSUDA, S., Laboratory of Veterinary Public Health, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka 020–8550, Japan.

MATERIALS AND METHODS

Animals: Seven week-old virgin female mice were purchased from Clea Japan, Inc (Tokyo), and were acclimated to the environment for 7 days. They were mated at the age of 8 to 10 weeks, and successfully copulated females were used in this study. The diet for this study was the commercially available pellet MF (Oriental Yeast Co., Ltd., Tokyo). Animals were given tap water. The diet and water were provided to the animals *ad libitum*. The animal room was kept under the following conditions: temperature of 22 ± 2°C, relative humidity of 20–40% and 12 hr light-dark cycle. All procedures were approved by the Animal Research Committee, Faculty of Agriculture, Iwate University, and were conducted under Guidelines for animal experiment in Iwate University.

Chemicals: Ethanol, tetraethylthiuram disulfide (disulfiram, DSF), carboxymethyl cellulose sodium (CMC) were obtained from Wako Pure Chemical Industries, Ltd., Osaka. Ethanol was diluted to 20 v/v% with distilled water. DSF was suspended with 0.5 w/v% CMC, to prepare 200 and 400 mg/kg dosing suspensions.

Regular (GP-42) and low-melting point (LGT) agar were purchased from Nacalai-tesque, Inc., Tokyo. They were diluted to 1 or 2 w/v%, respectively, with physiological saline.

All other chemicals were mainly purchased from Wako Pure Chemical Industries, Ltd., Osaka. The fully frosted or super frost slide glasses were purchased from Matsunami Glass, Osaka.

Chemical treatment and observation: Eight to 10 week-old females were caged with males overnight, and were checked for the presence of the vaginal plug in the next morning. The day when vaginal plug was confirmed was designated as Day 0 of gestation. On Day 7 of gestation, 4 successfully copulated females were assigned to each treatment and non-treated control group (4, 8, 12 and 24 hr). In our previous mouse Comet assays, we observed no significant differences in mean migration between vehicle control groups and corresponding untreated groups at any time for any organs [15–18]. The results enabled us to use untreated control animals as concurrent control animals. Any clinical changes were observed and recorded. The dose and the administration route of ethanol were based on the study, which reported that 4 g/kg orally administered ethanol caused DNA damage in rat brain [23]. There are some reports showing that administration of 200 mg/kg DSF significantly inhibited the acetaldehyde metabolism in mice [9, 11, 19]. The animals were orally exposed to either 20 v/v% ethanol at 2, 4 or 8 g/kg, or DSF in 0.5 w/v% CMC at 200 or 400 mg/kg. Furthermore, 200 or 400 mg/kg DSF was administered before 4 g/kg ethanol treatment. The methods were described below.

(i) Single oral doses of ethanol (4 and 8 mg/kg) were administered to animals in 4 groups. Four and 8 hr after administration, animals were euthanazed by cervical dislocation and their maternal organs/tissues (brain, lung, liver, kidney and mucous membranes of stomach, colon and urinary bladder) and embryos (2 to 4 embryos per dam, unless otherwise stated) were removed.

(ii) Based on the results of the experiment above, single oral doses of ethanol (2, 4 and 8 g/kg) were administered to animals in 12 groups. Four, 8, 12 and 24 hr after the administration, animals were euthanazed and their brain, lung, liver and embryos were removed.

(iii) DSF at the dose of 200 or 400 mg/kg was administered to the animals in other 4 groups. Eight and 12 hr after the administration, animals were euthanized and their maternal organs/tissues and embryos were removed as the same manner.

(iv) To detect the effect of acetaldehyde, pre-administration of 200 or 400 mg/kg DSF was performed 4 hr before the administration of 4 g/kg ethanol. Four and 8 hr after the administration of ethanol, the maternal organs/tissues and embryos were removed as the same manner.

Any changes in size, color and texture of all organs/tissues and embryos were recorded in each animal.

Comet assay: The organs/tissues and embryos were minced, suspended at 1 m*l*/g in chilled homogenizing buffer containing 0.075 M NaCl and 0.024 M Na_2EDTA, and gently homogenized at 500–800 rpm using a potter-type homogenizer. To obtain nuclei, the homogenate was centrifuged at 700 × g for 10 min at 0°C, and the precipitate was re-suspended in chilled homogenizing buffer at 1 g/m*l*. Seventy-five μl agarose GP-42 was quickly layered on a fully frosted slide (Matsunami Glass, Osaka) and covered with another slide. The slide sandwiches were placed to allow the agarose to the gel. The nuclear preparation was mixed 1:1 (v/v) with 2% agarose-LGT, and 75 μl of the mixture was quickly layered in the same manner after removal of the covering slide. Finally, another 75 μl agarose GP-42 was quickly layered on top. The slides were immediately placed in a chilled lysing solution (pH 10) of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma, 1% Sarkosyl, 10% DMSO, and 1% Triton X-100 and kept at 0°C in the dark for 1 to 24 hr.

The slides were placed on a horizontal gel electrophoresis platform and covered with chilled alkaline solution made up of 300 mM NaOH and 1 mM Na_2EDTA (pH 13). The slides were left in the solution in dark for 10 min, and then the DNA was electrophoresed at 0°C in the dark for 15 min at 1 V/cm and approximately 250 mA. The slides were gently rinsed in 400 mM Trisma (pH 7.5) to neutralize the excess alkali. Each slides was stained with 50 μl of 20 μg/m*l* ethidium bromide and covered with a coverslip.

Examination of the nuclei and statistical analysis: Fifty cells on each slide per organ/tissues or embryos from each animal were examined and photographed (black and white, 400 ASA, Fuji Film) in a fluorescence microscope (Olympus, at 200 × magnification) equipped with an excitation filter of 515–560 nm and barrier filter of 590 nm. The length of the whole comet ('length') and the diameter of the head ('diameter') were measured. Since broken DNA fragments stream further from the nucleus than intact DNA, the difference between length and diameter (migration) were calculated as the index of the DNA damage.

We compared the average of four mean migration distances per group (the unit of measure was the mouse). The migration distance was subjected to F-test for homogeneity of variance between 2 groups. Homogeneous data were analyzed by Student's *t*-test, and heterogeneous data by Mann-Whitney test, to assess the significant difference between 2 groups. As for statistical analysis among 3 groups, the data were first analyzed by one-way analysis of variance (ANOVA), and significant data were analyzed by Dunnett's multiple comparison test for significant differences. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Clinical findings: There were no dead animals in each group.

No clinical findings were observed in 2 g/kg groups.

Slight hyperpnea and ataxic gait was observed in 10 min after the administration of 4 g/kg ethanol in all animals. These findings were continually observed for about 2 hr

after the administration. No clinical findings were observed at 4, 8, 12 or 24 hr after administration in 4 g/kg groups.

When 8 g/kg ethanol was orally administered, decrease in locomotor activity, abnormal gait and hyperpnea were observed in 5 min after the treatment. Then, lateral position and loss of righting reflex were observed in all animals. Hypopnea, hypothermia, cyanosis and stupor lasted for 12 hr after the onset of the symptoms. No clinical findings were observed at 24 hr after 8 g/kg ethanol treatment in 3 of 4 animals, but 1 animal was still in stupor.

No death or clinical findings were observed in the single oral treatment of DSF.

When 4 g/kg ethanol was administered 4 hr after DSF treatment, similar clinical findings were observed as the single treatment of 4 g/kg ethanol, and there was no difference in recovery.

Necropsy findings: There were no gross abnormalities in any organs/tissues or embryos in 2 g/kg ethanol-treated groups. At 24 hr after the treatment of 4 and 8 g/kg ethanol, embryos were about half in size compared with the 24 hr-control group. Diluted lumen of stomach, liquid contents in jejunum and ileum were observed at 24 hr after 8 g/kg ethanol treatment. No gross abnormalities were observed in any other organs/tissues in 4 or 8 g/kg groups.

There were no gross abnormalities in any organs/tissues or embryos in the single administration of DSF groups, or DSF and ethanol-treated groups.

DNA migration: No significant differences were observed between each control group (4, 8, 12 and 24 hr). Each time control group was subjected to statistical analysis between control and each corresponding group treated with ethanol or DSF.

As the results of the experiment (i), significant increases of DNA damage were observed in brain, lung and embryos in 4 and 8 g/kg ethanol-treated groups. Significant increases of DNA damage were sporadically observed in kidney, mucus membranes of stomach, colon, and urinary bladder in 4 g/kg ethanol-treated groups. No significant difference was observed in the liver after ethanol treatment (Table 1).

Based on the results above, DNA migration in brain, lung, liver and embryos were observed at 4, 8, 12 and 24 hr after 2, 4 and 8 g/kg ethanol treatment, to detect the dose-dependency and the prolongation of DNA damage.

As for brain, which is considered as the target organ of ethanol toxicity, significant increases of DNA damage were observed at 4 to 12 hr after 4 and 8 g/kg ethanol-treated groups. Significant increases of DNA damage continued to 24 hr after 8 g/kg ethanol treatment. DNA damage peaked at 4 hr after the treatment. No significant increase of DNA damage was observed in 2 g/kg ethanol-treated groups (Fig.1A).

Lung is not generally considered as the target organ of ethanol toxicity, but significant increase of DNA damage was observed in experiment (i). Thus, we further investigated the effect of ethanol treatment. As the result, significant increases of DNA damage were observed at 4 and 8 hr after 4 and 8 g/kg ethanol treatment. In 8 g/kg groups, significant increase of DNA damage was also observed at 24 hr after the treatment. The DNA damage peaked at 4 hr after the treatment. No significant increases of DNA damage were observed in 2 g/kg ethanol-treated groups (Fig. 1B).

Liver is generally considered as the target organ of ethanol toxicity, but no significant increases of DNA damage were observed in any treatment of ethanol (Fig. 1C).

In embryos, the target of the ethanol toxicity, significant increases of DNA damage were observed at 4 to 12 hr after 4 and 8 g/kg ethanol treatment. In 8 g/kg groups, significant increase of DNA damage continued to 24 hr after the treatment. The DNA damage in embryos peaked at 8 hr after the treatment. No significant increases of DNA damage were observed in 2 g/kg ethanol-treated groups (Fig. 1D).

In 2 g/kg ethanol-treated groups, the DNA damage did

Table 1. DNA migration in maternal organs/tissues and embryos of pregnant mice treated with ethanol

Sampling Time (hr)	Dose (g/kg)		Animal Number	Migration (μm)							
				Brain	Lung	Liver	Kidney	Stomach	Colon	Urinary Bladder	Embryo
4	0	Mean	4	0.16	1.79	0.30	0.36	6.65	5.84	1.69	0.10
		SE		0.11	0.21	0.30	0.08	0.44	0.77	0.35	0.10
	4	Mean	4	1.97*	5.86*	0.30	1.48*	7.90	7.30	1.44	0.69*
		SE		0.69	1.08	0.23	0.45	1.59	1.42	0.52	0.16
	0	Mean	4	0.39	1.69	0.30	0.41	6.51	6.20	1.48	0.21
		SE		0.20	0.29	0.30	0.06	0.47	0.88	0.24	0.12
	8	Mean	4	2.41*	3.59**	0.39	0.91	8.46	7.59	3.61	1.07*
		SE		0.66	0.35	0.28	0.32	0.72	1.29	0.80	0.24
8	0	Mean	4	0.16	1.76	0.36	0.30	6.60	5.63	1.38	0.10
		SE		0.11	0.23	0.29	0.12	0.45	0.78	0.25	0.10
	4	Mean	4	0.69*	5.24**	0.14	0.86	12.53**	13.06**	4.04**	2.11*
		SE		0.15	0.84	0.14	0.22	0.95	1.41	0.51	0.70
	0	Mean	4	0.23	1.86	0.71	0.46	6.59	6.61	1.57	0.10
		SE		0.10	0.22	0.35	0.25	0.44	0.79	0.26	0.10
	8	Mean	4	1.16*	3.14*	0.79	2.91	11.10	9.09	2.91	5.78*
		SE		0.57	0.38	0.28	0.97	1.90	1.58	0.93	0.93

Significant difference; *: $p<0.05$, **: $0.001<p<0.01$.

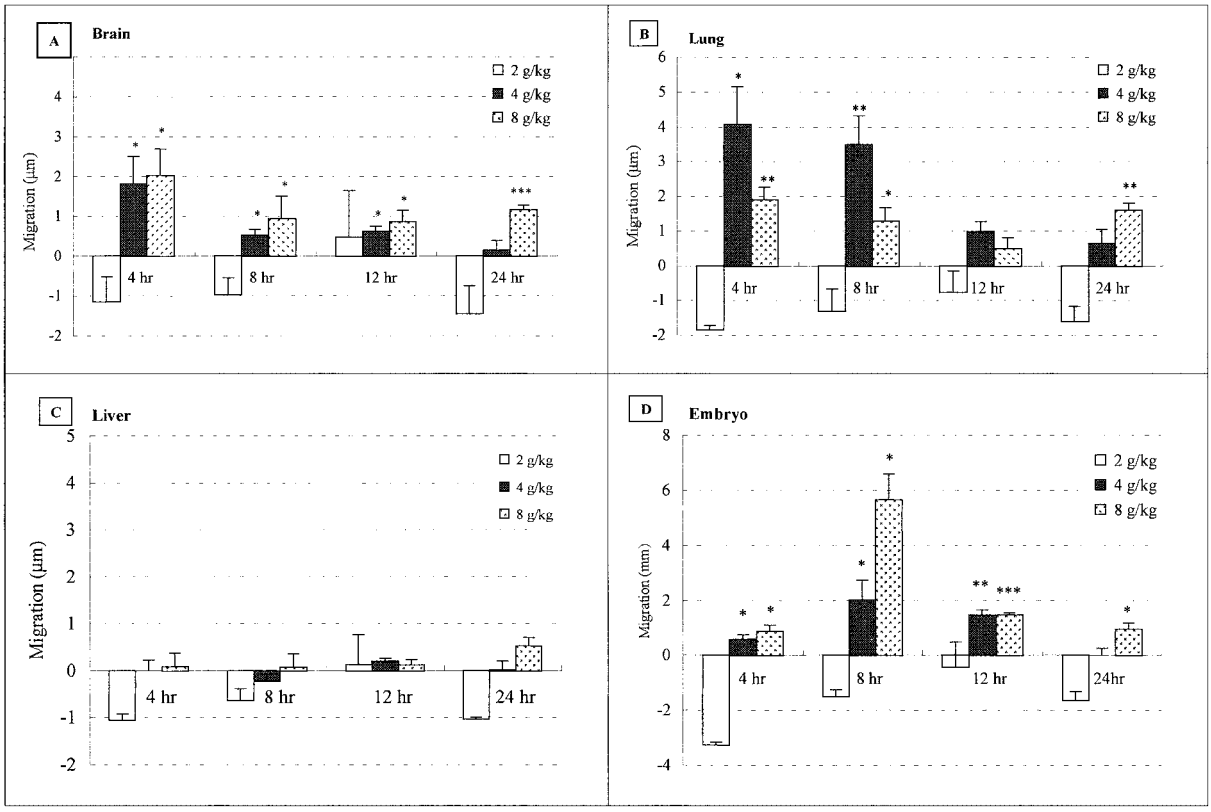

Fig. 1. DNA migration in maternal organs and embryos of pregnant mice treated with ethanol. Each data is shown as the difference between control and treated group. Significant difference; *:p<0.05, **:0.001<p<0.01, ***: p<0.001.

Table 2. DNA migration in maternal organs/tissues and embryos of pregnant mice treated with disulfiram

Sampling Time (hr)	Dose (mg/kg)		Animal Number	Migration (μm)							
				Brain	Lung	Liver	Kidney	Stomach	Colon	Urinary Bladder	Embryo
8	0	Mean	4	0.39	1.72	0.43	0.53	5.94	6.60	1.26	0.40
		SE		0.20	0.28	0.28	0.07	0.64	0.62	0.22	0.15
	200	Mean	4	0.34	3.89	0.56	0.90	8.96	7.87	2.79	0.63
		SE		0.27	1.01	0.10	0.23	1.64	0.50	0.42	0.31
	400	Mean	4	0.20	1.63	0.04	0.67	10.24	7.67	1.91	0.54
		SE		0.20	0.80	0.04	0.40	3.54	1.30	0.92	0.32
12	0	Mean	4	0.26	0.90	0.00	0.17	6.22	5.51	1.83	0.11
		SE		0.22	0.31	0.00	0.11	0.28	1.30	0.46	0.11
	200	Mean	4	0.30	2.54	0.31	0.66	5.67	5.27	1.91	0.44
		SE		0.30	0.87	0.22	0.26	1.01	0.42	0.22	0.35
	400	Mean	4	0.04	1.53	0.09	1.26	10.39	8.34	3.46	0.70
		SE		0.04	0.46	0.09	0.30	1.30	0.14	0.91	0.59

Significant difference; *: p<0.05.

not increase in any organs or embryos, rather tended to decrease compared with those of control groups. No significant difference was observed between 2 g/kg ethanol-treated groups and control.

Compared with 4 g/kg ethanol-treated groups, the degree of DNA damage increased in embryos at 8 hr after 8 g/kg ethanol treatment, but not in brain or lung.

Neither 200 nor 400 mg/kg DSF caused DNA damage in any samples examined (Table 2).

Ethanol did not increase DNA damage in any organs/tissues or embryos in the pre-treatment of DSF except for colon and urinary bladder at 8 hr after ethanol treatment in the presence of 200 mg/kg DSF (Table 3).

To investigate the effect of pre-treatment of DSF, the increased DNA migration detected in 4 g/kg ethanol treated group was compared with 200 or 400 mg/kg DSF pre-

Table 3. DNA migration in maternal organs/tissues and embryos in pregant mice treated with disulfiram and ethanol

Sampling Time (hr)[a)]	Dose			Animal	Migration (μm)							
	Ethanol (g/kg)	Disulfiram[b)] (mg/kg)		Number	Brain	Lung	Liver	Kidney	Stomach	Colon	Urinary Bladder	Embryo
4	0	200	Mean	4	0.34	3.89	0.56	0.90	8.96	7.87	2.79	0.63
			SE		0.27	1.01	0.10	0.23	1.64	0.50	0.42	0.31
	4	200	Mean	4	1.31	4.13	2.85	0.72	10.69	12.12	6.92	0.84
			SE		0.41	1.73	1.65	0.57	4.05	3.37	3.26	0.18
	0	400	Mean	4	0.20	1.63	0.04	0.67	10.24	7.67	1.91	0.54
			SE		0.20	0.80	0.04	0.40	3.54	1.30	0.92	0.32
	4	400	Mean	4	1.16	4.60	0.00	0.39	11.33	10.19	4.89	0.69
			SE		0.72	1.51	0.00	0.27	2.00	1.80	1.83	0.58
8	0	200	Mean	4	0.30	2.54	0.31	0.66	5.67	5.27	1.91	0.44
			SE		0.30	0.87	0.22	0.26	1.01	0.42	0.22	0.35
	4	200	Mean	4	0.07	3.46	0.17	0.79	9.33	13.16 *	4.51 *	0.37
			SE		0.07	0.94	0.17	0.47	2.34	1.88	0.82	0.23
	0	400	Mean	4	0.04	1.53	0.09	1.26	10.39	8.34	3.46	0.70
			SE		0.04	0.46	0.09	0.30	1.30	0.14	0.91	0.59
	4	400	Mean	4	1.17	5.20	0.10	0.51	13.53	16.59	5.39	1.51
			SE		0.59	1.82	0.10	0.51	1.64	4.26	2.28	0.84

a): Sampling time (hr) is the time after ethanol treatment.
b): Disulfiram was administered 4 hr before ethanol treatment.
Significant difference; *: $p<0.05$.

treated groups. As the result, no significant increases were observed among single treatment of 4 g/kg ethanol, 200 or 400 mg/kg DSF pre-treated groups. Furthermore, significant decrease of DNA damage was observed in the brain at 8 hr after 4 g/kg ethanol treatment with 200 mg/kg pre-treatment of DSF. DNA damage induced by ethanol tended to be depressed in lung and embryos in DSF pre-treated groups (Fig. 2).

As the result, pre-treatment of DSF did not increase the DNA damage induced by ethanol. Furthermore, the DNA damage induced by ethanol was depressed by pre-treatment of DSF.

DISCUSSION

To detect the DNA damage induced by ethanol, the alkaline single cell gel electrophoresis (Comet) assay was performed in pregnant mice with multiple organs.

We administered ethanol to pregnant mice on Day 7 of gestation, since it is considered as organogenic period of mice [25]. In this study, significant increases of DNA damage were observed in brain, lung, and embryos of pregnant mice when administered 4 and 8 g/kg ethanol. Twice as low as 4 g/kg ethanol did not cause any DNA damage, and, twice as high as 4 g/kg ethanol only increased the duration of DNA damage without distinct increase in the degree of the damage. No significant DNA damage was observed in the liver.

There were reports showing that significant increase of DNA damage was observed in rat brain administered 4 g/kg ethanol [23], and acute ethanol exposure induced DNA damage in cultured neurons [20]. Excess chronic intake of ethanol causes severe damages on the brain such as alcoholic cellebellar atrophy [6]. The DNA damage in the brain detected in this study might be related to ethanol neurotoxicity.

It is well known that large consumption of alcohol during pregnancy causes fetal alcohol syndrome (FAS). There are many studies about ethanol teratogenicity, especially about the effects of chronic alcohol consumption. Binge drinking during pregnancy is also considered as important factor of FAS, as possible human teratogen [2, 13]. We administered ethanol to pregnant mice on Day 7 of gestation. Some reports show the teratogenic effects of ethanol on mice fetuses, especially external anomalies, when administered to dams on the same period [25]. In this study, the size of the embryos at 24 hr after ethanol treatment was apparently smaller than those in control groups, it is considered that ethanol treatment induced growth inhibition in mice embryos. Since embryos on Day 7 of gestation are very small, we cannot say which part of the embryos were damaged by ethanol in this study. The DNA damage in embryos detected in this study might have some effects on ethanol embryotoxicity or FAS.

No significant increases of DNA damage were detected in liver and stomach, which are considered as the target organ of ethanol toxicity. On the other hand, significant increase of DNA damage was detected in the lung when administered 4 and 8 g/kg ethanol. Lung is not generally considered as the target organ of ethanol. Epidemiological studies suggest there are some kinds of relation between lung cancer and chronic alcohol consumption [1]. Since DNA damage is one of the important factors of carcinogenicity, the results obtained in this study suggest that the DNA damage in the lungs might have effects on lung cancer. But there are no reports about relation between lung

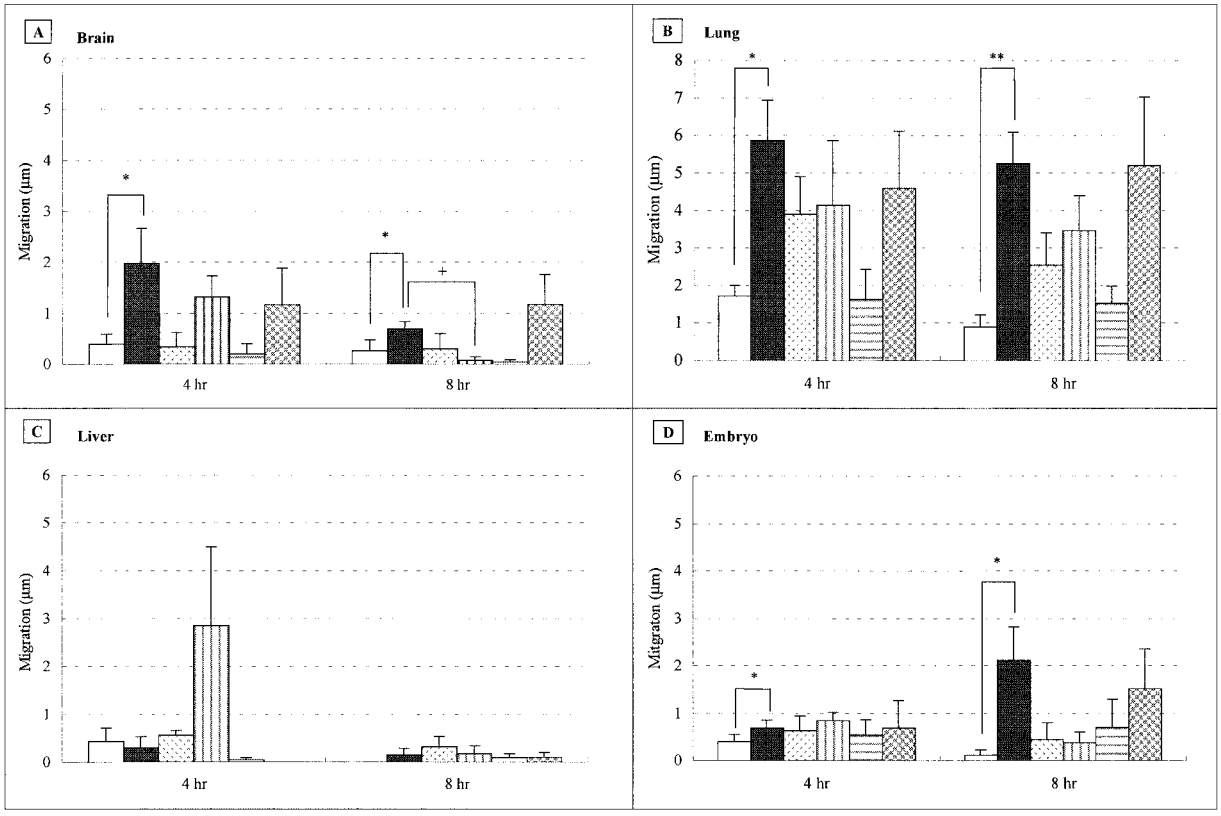

Fig. 2. The effects of pre-treatment of disulfiram on DNA damge induced by 4 g/kg ethanol in maternal organs and embryos in pregnant mice. Hr is the time after ethanol treatment. Significant difference between control and ethanol or disulfiram treatment groups; *: $p<0.05$, **: $0.001<p<0.01$. Significant difference between ethanol alone and disulfiram pre-treated ethanol (200 or 400 mg/kg disulfiram); +: $p<0.05$. □: Non-treatment. ■: Ethanol 4 g/kg. ⣿: Disulfiram 200 mg/kg. ▥: Pre-treatment of disulfiram 200 mg/kg and ethanol 4 g/kg. ▤ : Disulfiram 400 mg/kg. ▨: Pre-treatment of disulfiram 400 mg/kg and ethanol 4 g/kg .

cancer and acute ethanol exposure in experimental animals as far as we know. The DNA damage induced by ethanol in the lung and its role in lung cancer should be examined further.

The degree of DNA damage observed in embryos increased at 8 hr after 8 g/kg ethanol treatment, but not in brain or lung. Significant increases of DNA damage induced by ethanol prolonged in brain, lung and embryos in 8 g/kg ethanol-treated groups. These results suggest that the DNA damages induced by ethanol are prolonged dose-dependently, and continuous DNA damage might cause more errors in DNA repair. Ethanol-induced DNA damage and the failure to repair the damaged DNA are considered as the factors in alcohol related-brain damage [6].

In this study, DNA damage did not increase in 2 g/kg ethanol treatment. There is a report showing that low dose of ethanol can inhibit DNA strand break *in vitro* [4]. But the mechanism to reduce DNA damage detected in this study is not clear.

Acetaldehyde, metabolite of ethanol is considered as DNA crosslinker [8, 26]. The mechanism of DNA damage caused by ethanol is not well studied. However, some reports show that acetaldehyde induces DNA-protein crosslink in brain, liver and other organs in rat [12], and is considered to play an important role in ethanol-induced DNA damage [12, 26]. To detect the effect of acetaldehyde on the DNA damage induced by ethanol, we administered DSF 4 hr before ethanol treatment. As the result, DSF itself had no effect on DNA migration. DNA damage was not increased by pre-treatment of 200 or 400 mg/kg DSF. Furthermore, pre-treatment of DSF reduced DNA damage by ethanol in some organs. Theses results suggest that DSF has no effect to increase DNA damage. There are reports showing that DSF prevents the ethanol-induced lipid peroxidation in rat liver [14], and it inhibits DNA fragmentation [27]. The DNA damage induced by ethanol may not be the effect of acetaldehyde, but direct effect of ethanol or caused by some other factors.

We could not find any reports on the degree of resultant mutation after the DNA damage caused by ethanol. Although almost all of DNA damage would be repairable, it is generally considered that DNA damage caused by chemicals increase the possibility of mutation. We need further investigation on the relation between the carcinogenicity or teratogenicity of ethanol and DNA damages observed in this study.

ACKNOWLEDGMENTS. The authors are deeply grateful to people in Biosafty Research Center, Foods, Drugs and Pesticides (the genetic toxicology group and the reproductive toxicology group for technical supports and Dr. Kiyoshi Imai for scientific advices).

REFERENCES

1. Bendera, E.V., Freudenheim, J.L. and Vena, J.E. 2001. Alcohol consumption and lung cancer: a review of the epidemiologic evidence. *Cancer Epidemiol. Biomakers Prev.* **10**: 813–821.
2. Bishop, J.B., Witt, K.L. and Sloane, R.A. 1997. Genetic toxicities of human teratogens. *Mutat. Res.* **396**: 9–43.
3. Blakley, P. M. and Scott, W. J. Jr. 1984. Determination of the proximate teratogen of the mouse fetal alcohol syndrome. 2. pharmacokinetics of the placental transfer of ethanol and acetaldehyde. *Toxicol. Appl. Pharmacol.* **72**: 364–371.
4. Cao, Z. and Li, Y. 2004. Potent inhibition of peroxynitrite-induced DNA strand breakage by ethanol: possible implications for ethanol-mediated cardiovascular protection. *Pharmacol. Res*. **50**: 13–19.
5. Klaassen, D.K. 2001. Casarett and Doull's Toxicology 6th ed. McGraw-Hill, New York.
6. Harper, C. and Matsumoto, I. 2005. Ethanol and brain damage. *Curr. Opin. Pharmacol.* **5**: 73–78.
7. IARC monographs on the evaluation on carcinogenic risks to humans: Alcohol drinking. 1988. International agency for Research on Cancer. 44.
8. IARC monographs on the evaluation on carcinogenic risks to humans: Acetaldehyde. 1999. International Agency for Research on Cancer. 71.
9. Kurata, N., Kemper, R., Hurst, H.E. and Waddell, W.J. 1990. Inhibition of the metabolism of ethyl carbamate by acetaldehyde. *Drug-Metab. Dispos.* **18**: 504–507.
10. Larkby, C. and Day, N., 1997. The effects of prenatal alcohol exposure. *Alcohol Health Res. World.* **21**: 192–198.
11. Mahmound, M. I., Potter, J.J., Colvin, O. M., Hilton, J. and Mezey, E. 1993. Effect of 4-(diethylamino)benzaldehyde on ethanol metabolism in mice. *Alcohol Clin. Exp. Res.* **17**: 1223–1227.
12. Niemela, O. 2001. Distribution of ethanol-induced protein adducts *in vivo*: relationship to tissue injury. *Free Radic. Biol. Med.* **31**: 1533–1538.
13. Nulman, I., Rovet, J., Kennedy, D., Wasson, C., Gladstone, J., Fried, S. and Koren, G. 2004. Binge alcohol consumption by non-alcohol-dependent women during pregnancy affects child behavior, but not general intellectual functioning; a prospective controlled study. *Arch. Women Ment. Health* **7**: 173–181.
14. Pron'ko, P.S., Kuz'mich, A.B. and Abakumov, G.Z. 1999. Effect of alcohol intoxication and aldehyde dehydrogenase inhibitors on lipid peroxidation in rat liver. *Ukr. Biokhim.* **71**: 79–83.
15. Sasaki, Y. F., Tsuda, S., Izumiyama, F. and Nishidate, E. 1997. Detection of chemically induced DNA lesions in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow) using the alkaline single cell gel electrophoresis (Comet) assay. *Mutat.Res.* **388**: 33–44.
16. Sasaki, Y. F., Izumiyama, F., Nishidate, E., Matsusaka, N. and Tsuda, S. 1997. Detection of rodent liver carcinogen genotoxicity by the alkaline single-cell gel electrophoresis (Comet) assay in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow). *Mutat. Res.* **391**: 201–214.
17. Sasaki, Y. F., Nishidate, E., Izumiyama, F., Matsusaka, N. and Tsuda, S. 1997. Simple detection of chemical mutagens by the alkaline single-cell gel electrophoresis (Comet) assay in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow). *Mutat. Res.* **391**: 215–231.
18. Sasaki, Y. F., Nishidate, E., Izumiyama, F., Watanabe-Akanuma, M., Kinae, N., Matsusaka, N. and Tsuda, S. 1997. Detection of *in vivo* genetoxicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) by the alkaline single cell gel electrophoresis (Comet) assay in multiple mouse organs. *Mutat. Res.* **393**: 47–53.
19. Sharkawi, M. 1980. Inhibition of alcohol dehydrogenase by disulfiram; possible relation to the disulfiram—ethanol reaction. *Life Sci.* **27**: 1939–1945.
20. Signorini-Allibe, N., Gonthier, B., Lamarche, F., Eysseric, H. and Barret, L. 2005. Chronic consumption of ethanol leads to substantial cell damage in cultured rat astrocytes in conditions promoting acetaldehyde accumulation. *Alcohol Alcohol.* **40**: 163–171.
21. Simm, B. and Murdoch, R.N. 1990. The role of acetate in alcohol-induced alterations of uterine glucose metabolism in the mouse during pregnancy. *Life Sci.* **47**: 1051–1058.
22. Singh, N.P., Mccoy, M.T., Tice, R.R. and Schneider, E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **175**: 184–191.
23. Singh, N.P., Lai, H. and Khan, A. 1995. Ethanol-induced single-strand DNA breaks in rat brain cells. *Mutat. Res.* **345**: 191–196.
24. Tsuda, S., Kosaka, Y., Matsusaka, N. and Sasaki, Y. F. 1998. Detection of pyrimethamine-induced DNA damage in mouse embryo and maternal organs by the modified alkaline single cell gel electrophoresis assay. *Mutat. Res.* **415**: 69–77.
25. Webster, W. S., Walsh, D.A., McEwen, S. E. and Lipson, A.H. 1983. Some teratogenic properties of ethanol and acetaldehyde in C57BL/6J Mice: Implications for the study of the fetal alcohol syndrome. *Teratology* **27**: 231–243.
26. Xi, ZG., Chao, F.H., Yang, D.F., Sun, Y.M., Li, G.X., Zhan, H.S., Zhang, W., Yang, Y.H. and Liu, H.L. 2004. The effects of DNA damage induced by acetaldehyde. *Huan. Jing. Ke. Xue.* **25**: 102–105.
27. Zhao, A., Wu, Z.Q., Pollack, M., Rollewagen, F.M., Hirszel, P. and Zhou, X. 2000. Disulfiram inhibits TNF-alpha-induced cell death. *Cytokine* **12**: 1356–1367.