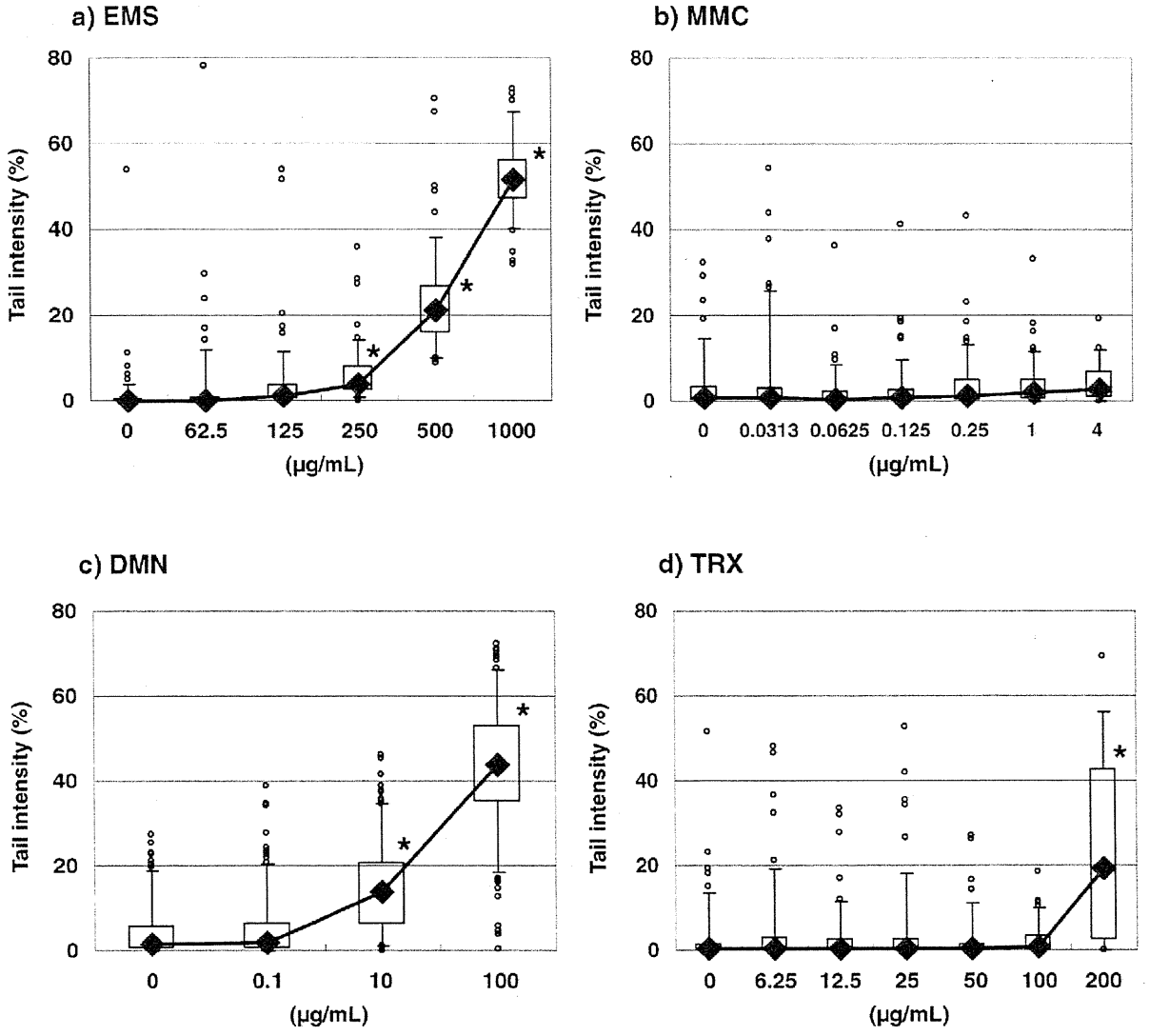

Fig. 2. Response of DNA damage induced in CHL cells after treatment with the test chemicals. CHL cells were treated with EMS (a), MMC (b), or TRX (d) for 4 h without metabolic activation, and were treated with DMN (c) for 6 h with metabolic activation. DNA damage was measured by the comet assay as tail intensity. Each data point in box plots represents the mean (± SEM) of 100 cells treated with EMS (a), MMC (b), or TRX (d) and of 200 cells treated with DMN (c) for each dose. *Significant increase from solvent control by Dunnett's multiple comparison test ($P<0.05$).

2b) show no significant increase in tail intensity up to 4 μg/mL. There were only 35 analyzable images at the highest dose of 4 μg/mL. MMC, which is a known interstrand cross-linker, did not increase tail intensity, and was considered to have induced DNA-DNA cross-links, preventing separation of the DNA duplex (5,6). MMC is reported to be positive for the Ames assay using *Escherichia coli* WP2, *in vitro* micronucleus test using human lymphocytes, *in vivo* micronucleus test, and MLA assay using L5178Y cells by TOXNET.

DMN: DMN was examined at 7 dose levels, 0.1, 10, 100, 500, 1000, 3000, and 5000 μg/mL with metabolic activation. The results of the comet assay (Fig. 1c and 2c) show a significant dose-related increase in tail intensity at 10 μg/mL and greater. At dose levels of 500 μg/mL and greater, the comet images could not be analyzed because only hedgehogs were observed. The assay for DMN without metabolic activation was performed concomitantly at the same dose levels. The results of the assay showed no significant increase in tail intensity at up to 5000 μg/mL (data not shown). DMN is reported to be positive for the Ames assay with metabolic activation, *in vitro* sister-chromatid exchange (SCE) with metabolic activation using human lymphocytes, *in vivo* micronucleus test, and MLA assay with metabolic activation using L5178Y cells by TOXNET. Winter *et al.* demonstrated that DMN clearly induced tail intensity in an *in vitro* comet assay using human hepatocellular lines

expressing metabolic enzymes (7).

TRX: TRX was examined at 6 dose levels, 6.25, 12.5, 25, 50, 100, and 200 μg/mL without metabolic activation. The results of the comet assay (Fig. 1d and 2d) show no significant increase in tail intensity at up to 100 μg/mL. At 200 μg/mL, the highest dose level, only 17 comet images were able to be analyzed and a significant increase in tail intensity was noted. This was considered due to an irrelevant positive response, because severe cytotoxicity, determined by dye-exclusion assay (trypan blue), was noted at only this dose. TRX is a detergent and an absolutely non-genotoxic agent (8).

In conclusion, the simple *in vitro* comet assay method using MAS-coat type slide glasses can clearly detect genotoxic chemicals other than DNA cross-linking agents such as MMC. Because the original comet assay also is unable to detect DNA cross-linking agents, the failure is a feature of the comet assay, and not due to the methodology (5,6,9). The sensitivity and reproducibility of the simple comet assay method must be equal to the conventional method, although it contributes to reduce labor and time. It could hold promise for the application to a high-throughput comet assay.

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Biological safety of neutral-pH hydrogen-enriched electrolyzed water upon mutagenicity, genotoxicity and subchronic oral toxicity

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Abstract

Hydrogen-dissolved water has been suggested to be effective for alleviating the oxidative stress. In the present study, neutral-pH hydrogen-enriched electrolyzed water (NHE-water; dissolved hydrogen: 0.90–1.14 parts per million [ppm]; oxido-reduced potential: −150 ~ −80 mV), which was prepared with a water-electrolysis apparatus equipped with a non-diaphragm cell and a highly compressed activated-carbon block, was evaluated for the mutagenic and genotoxic potentials, at concentrations up to 100% dose/plate, and for the subchronic toxicity. NHE-water did not induce reverse mutations in *Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537, and *Escherichia coli* strain WP2uvrA, in either the absence or presence of rat liver S9 for exogenous metabolic activation. Similarly, NHE-water did not induce chromosome aberrations in Chinese hamster lung fibroblast cells (CHL/IU), in short-term (6-hour) tests, with or without rat liver S9, or in a continuous treatment (24-hour) test. To evaluate the subchronic toxicity, Crj:CD(SD) specific pathogen free (SPF)-rats were administered with NHE-water at a dose of 20 mL/kg/day for 28 days via intragastric infusion. NHE-water-related toxic changes were not seen in terms of any items such as clinical symptoms, body weight, food consumption, urinalysis, hematology, blood chemistry, necropsy, each organ weight and histopathology. Thus, the no-observable-adverse-effect level (NOAEL) for NHE-water was estimated to be greater than 20 mL/kg/day under the conditions examined, demonstrating the consistency with the expected safety for a human with a body weight of 60 kg to drink the NHE-water up to at least 1.2 L/day.

Keywords

hydrogen-enriched electrolyzed water, mutagenicity, genotoxicity, subchronic oral toxicity

Introduction

Hydrogen has been noted as a novel antioxidant material. Inhalation of hydrogen gas markedly suppresses oxidative stress-related injuries such as post-ischemic reperfusion-induced brain (Ohsawa et al., 2007), myocardial injuries (Hayashida et al., 2008), hepatic injury in mice (Fukuda et al., 2007) and transplantation-induced intestinal graft injury in rats (Buchholz et al., 2008). Furthermore, aqueous solutions containing high concentrations of dissolved hydrogen also possess antioxidant effects. Hydrogen-dissolved solutions not only exert antioxidant activities against hydroxyl radicals (Ohsawa et al., 2007; Saitoh et al., 2008) and peroxynitrites (Ohsawa

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et al., 2007) in cell-free systems but also scavenge intracellular hydroxyl radical (Ohsawa et al., 2007) or decreases intracellular peroxides (Saitoh et al., 2008). And hydrogen-dissolved solutions show neuroprotective effects in neonatal hypoxia-ischemia rat (Cai et al., 2009), protective effect on oxidative stress-induced cell death (Ohsawa et al., 2007) and tumor-preferential clonal-growth-inhibition and tumor invasion (Saitoh et al., 2008).

In addition, it has been demonstrated that drinking of hydrogen-dissolved water decreased the ROS-induced urinary secretion of 8-hydroxydeoxyguanosine and hepatic formation of peroxidized lipid in rats (Yanagihara et al., 2005), the post-hypoxic-reoxygenation-induced ROS formation in vitamin C-depleted mice (Sato et al., 2008), chronic physical restraint stress-induced increases in oxidative stress markers (malondialdehyde and 4-hydroxy-2-nonenal in the brain) in mice (Nagata et al., 2009) and atherosclerosis in mice (Ohsawa et al., 2008). In the administration to humans, the improvement efficacy of hydrogen-dissolved water has been demonstrated in clinical trials for patients with type-2 diabetes or impaired glucose tolerance (Kajiyama et al., 2008).

Thus, although many reports have suggested that hydrogen and hydrogen-dissolved water are quite effective for alleviating the oxidative stress, little is known, as a great premise for the practice application, about the safety of hydrogen-dissolved water. In order to provide evidences of the safety of hydrogen-dissolved water, the mutagenicity, genotoxicity and subchronic oral toxicity were evaluated in the present study.

Materials and methods

Study design

These studies were performed in 2005 at the Biosafety Research Center, Foods, Drugs and Pesticides (582-2, Shioshinden, Iwata, Shizuoka 437-1213, Japan) in compliance with 'Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives' of the Japanese Ministry of Health and Welfare (Notification Eika No. 29, 22 March 1996), and in accordance with the principles for Good Laboratory Practice (GLP; Ministry of the Environment, Ministry of Health, Labour and Welfare, and Ministry of Economy, Trade and Industry, 2003), 'Law for the Protection and Management of Animals' [Law No. 105, 1 October 1973: hereinafter referred to as 'the animal protection law' (Law for the Protection and Management of Animals, 1982)], 'Standards Relating to the Care and Management of Experimental Animals' [Notice No. 6 of the Prime Minister's office, 27 March 1980: hereinafter referred to as 'the experimental animal standards' (Standards Relating to the Care and Management of Experimental Animals, 1982)], and the Guidance for Animal Testing at BSRC.

Test substances and chemicals

NHE-water was produced by a water-electrolysis apparatus Active BIO (Takaoka Electric MFG. Inc., Tokyo, Japan), which electrolyzed tap water in a non-diaphragm type cell with a super-flatness surface platinum-coated titanium electrode plate and once accumulated, the generated hydrogen in a high-pressure compressed activated-carbon block for producing NHE-water (Figure 1). Concentrations of dissolved molecular hydrogen (DH) in the solution were measured using a DH meter (DH-35A, DKK-Toa Inc., Tokyo, Japan), and oxido-reduced potential (ORP) and pH were measured by a pH/ORP meter (D-55, Horiba Inc., Kyoto, Japan), respectively. NHE-water had the following physical properties: pH 7.5–7.9, high DH (0.90–1.14 mg/L; concentrations immediately after preparation) and negative ORP (–150 to –80 mV) at 13–25°C. The typical data on temporal changes of dissolved hydrogen concentration (DH) of NHE-water were shown in Figure 1(B).

NHE-water was filtrated through 0.2 μm-pore filter and serially diluted with sterile water for injection (The Japanese Pharmacopoeia; Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) using a dilution factor 2 to make 4 or 5 concentrations for the bacterial mutation test or chromosome aberration test. For the bacterial reverse mutation test, the S9 fraction (lot no. RAA-520 produced in April 2005, Kikkoman Corp., Chiba, Japan, protein content; 24.86 mg/mL) was prepared from the livers of 7-week-old male Sprague-Dawley rats treated with phenobarbital and 5,6-benzoflavone. The positive-control substances were as follows 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF2), sodium azide (NaN_3), 9-aminoacridine hydrochloride (9-AA) and 2-aminoanthracene (2-AA) obtained from Oriental Yeast Co., Ltd (Tokyo, Japan).

For the chromosome aberration test, the S9 fraction (lot no. RAA-518, produced in March 2005 by Kikkoman Corp., protein content; 25.36 mg/mL) was prepared as previously described. Mitomycin C (MMC; Kyowa Hakko Kogyo Co., Ltd, Tokyo,

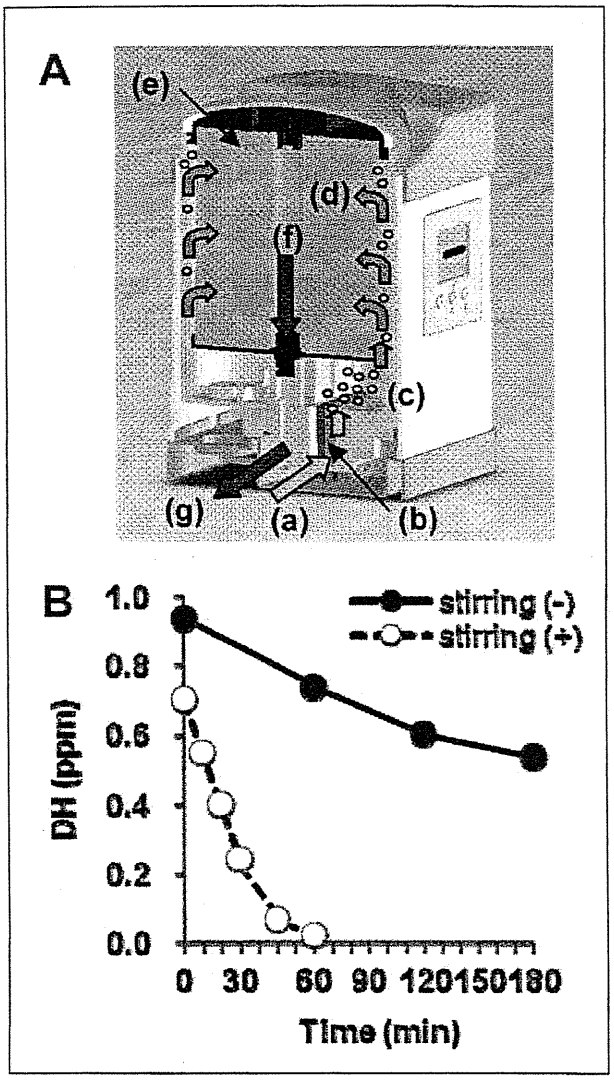

Figure 1. (A) A structure of an NHE-water production device. (a) Tap water inlet; (b) electrolysis tank equipped with three platinum-plating titanium electrodes; (c) electrolysis-produced gas; (d) electrolytically produced hydrogen gas is collected; (e) high-pressure- compressed activated-carbon block, (f) the hydrogen gas which is dissolved in water, is once stored up in a high-pressure compressed activated-carbon block and (g) NHE-water is taken out. (B) The temporal changes of dissolved hydrogen concentration (DH) in NHE-water. NHE-water was statically placed without stirring (stirring [–]) or gently stirred (stirring [+]) at the room temperature.

Japan) dissolved in sterile water for injection was diluted in physiological saline (The Japanese Pharmacopoeia; Otsuka Pharmaceutical Factory, Inc.). It was subdivided to 1 mL and stored frozen. This positive control was used at doses of 0.1 and 0.05 μg/mL for the 6- and 24-hour treatments (−S9), respectively. Cyclophosphamide (CP; Shionogi & Co., Osaka, Japan) dissolved in sterile water for injection was diluted with physiological saline. It was subdivided to 1 mL and stored frozen. This positive control was used in the presence of S9 at a dose of 12.5 μg/mL.

The 28-day toxicity study was performed with NHE-water and sterile water for injection as a control.

Bacterial reverse mutation test

NHE-water was tested in *Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537, and *Escherichia coli* strain WP2uvrA in the absence and presence of exogenous metabolic activation by rat liver S9, according to previously published methods (Ames, Durston et al., 1973; Ames, Lee et al., 1973; Ames et al., 1975; Ishidate, 1991; Japan Industrial Safety and Health Association, 1991; Yahagi, 1975). The *S. typhimurium* strains were received from Dr Bruce N Ames (University of California) on 9 September 1983, and the *E. coli* strain was provided by the National Institute of Health Sciences (Tokyo, Japan) on 16 March 1983. The composition of S9 mix used in this test was as follows: S9 0.1 mL, $MgCl_2$ 8 μmol, KCl 33 μmol, glucose-6-phosphate (G-6-P) 5 μmol, NADPH 4 μmol, NADH 4 μmol and sodium phosphate buffer (pH 7.4) 100 μmol in 1 mL of S9 mix. Based on the results of a concentration range–finding test (data not shown), the highest concentration in the mutagenicity test was established at 100%. The positive-control substances were AF2, 9-AA, 2-AA and NaN_3; a vehicle control (sterile water for injection) was also included. Tests were performed by the preincubation method. Before preincubation, 100 μL of the vehicle, test substance or the positive-control substance solution was added into 500 μL of sodium phosphate buffer (pH 7.4) in the -S9 assay or 500 μL of S9 mix in the +S9 assay, and then, 100 μL of bacterial suspension was also added. After a 48-hour incubation period, the number of revertants was counted using an automated colony analyzer (System Science Co.; CA-11). Two plates per concentration were prepared for the vehicle control (sterile water for injection), test substance treatment, positive- and negative-control groups. The results were judged to be positive when the mean number of revertants in each concentration increased to two-fold or more than the negative control, and reproducibility and concentration-dependent relationship were observed.

Chromosomal aberration test in CHL/IU cells

NHE-water was evaluated for its potential to produce chromosome aberrations in Chinese hamster lung fibroblast (CHL/IU) cells in vitro in the absence and presence of exogenous metabolic activation by rat liver S9 following 6-hour treatments and in the absence of S9 following 24-hour treatments according to the results of checking cell-doubling time and previously published methods (Evans, 1976; Ishidate, 1979; Ishidate, 1987; Ishidate and Odashima 1977; Matsuoka et al., 1979; Report of the Ad Hoc Committee of the Environmental Mutagen Society and the Institute for Medical Research, 1972). The cell subline passaged 27 times, under storage since it was supplied by the National Institute of Health Sciences on 15 November 1984, was cultured in Eagle's minimum essential medium (MEM; ASAHI Techno Glass Co., Ltd, Chiba, Japan) supplemented with 10% heat-inactivated calf serum (Invitrogen, CA, USA) in a CO_2 incubator (5% CO_2) at 37°C. Five milliliters of the cell suspension containing 8 × 103 cells/mL was seeded onto cell culture plate (60 mm; Sumitomo Bakelite Co., Ltd, Tokyo, Japan) and incubated for 3 days. Following incubation, 300 μL of the vehicle, test substance suspension, or the positive-control substance solution was added; 500 μL of S9 mix was also added to the +S9 assay. Following the incubation, 300 μL of the vehicle, test substance suspension or the positive-control substance solution was added into 2.7 mL (–S9 assay) or 2.2 mL (+S9 assay) of the culture medium and then, 500 μL of S9 mix was also added to the +S9 assay system. The composition of S9 mix was as follows: S9 0.3 mL, $MgCl_2$ 5 μmol, KCl 33 μmol, G-6-P 5 μmol, NADP 4 μmol, HEPES buffer (pH 7.2) 4 μmol and distilled water 0.1 mL in 1 mL of S9 mix. In the short-term treatment assays, the culture medium was removed after 6 hours and the cells were washed with Dulbecco's phosphate-buffered saline (Sigma-Aldrich, MO, USA). Three milliliters of fresh culture medium was added, and the cells were incubated for another 18 hours; in the continuous treatment assay, cells were incubated for 24 hours after the addition of the vehicle, test substance suspension or positive-control substance solution.

A growth inhibition test using the crystal violet method was performed to determine the concentration range of the test substance for the chromosomal aberration test (data not shown). Since no toxic effects were noted in either a 6-hour treatment or a 24-hur treatment, the highest concentration for the chromosomal aberration test was established at 100%. In addition, three lower concentrations were included (12.5, 25 and 50%). Two plates per concentration were prepared for the vehicle control (sterile water for injection), test-substance treatment and the positive-control groups. Two chromosomal slides per plate were prepared after incubation with the vehicle control, test substance or positive-control substance as follows: colcemid solution (Invitrogen) was added to the treated cells at a final concentration of 0.2 μg/mL, 2 hours before slide preparation to inhibit mitosis at metaphase. The culture medium was transferred into a centrifuge tube. The cells were stripped from the plate using 0.25% trypsin (Invitrogen), and the cell suspension was added to the culture medium in the centrifuge tube. Following centrifugation at 1000 rpm for 5 min, the culture medium was removed and the cells were subjected to hypotonic treatment in 5 mL of 75 mmol/L KCl for 16 min at 37°C. The hypotonic solution was removed by centrifugation and the cells were fixed in a cold (4°C) fixative solution (3:1 v/v methanol/acetic acid), washed twice and resuspended in a small volume of fresh fixative solution. One drop of the suspension was placed on each slide glass. The slides were allowed to dry and stained for 12 min with 1.2% Giemsa solution (Merck KGaA, Darmstadt, Germany) diluted with 1/100 mol/L sodium phosphate buffer solution (pH 6.8; Merck KGaA) and then rinsed with water and dried.

Cell growth inhibition was determined at the preparation of chromosomal slides with an ATP spectrophotometer (Lumitester C-100LU; Kikkoman Corp.) for the negative-control, treatment and positive-control groups. Cell suspension after hypotonic treatment (50 μL) was transferred into a small test tube containing 2 mL of 1% Tween 80 solution and mixed. This mixture was left still for about 20 min and 100 μL of luminescent reagent of reagent kit for ATP determination (Lucifer 250; Kikkoman Corp.) was transferred into test tube for determination. The rate to the relative light unites (RLU) in the negative-control group (cell survival rate) was calculated for each dose and determined as cell growth inhibition.

Microscopic examination was performed on the three observable doses for observation of chromosome aberrations owing to 25% to 100% for all assays. After all the slides had been coded, they were examined under code. One hundred metaphases per plate (200 cells/dose) were examined microscopically at a magnification of 600. The types of structural aberrations were classified into six groups: gap (gap), chromatid break (ctb), chromosome break (csb),

chromatid exchange (cte), chromosome exchange (cse) and others (oth). A gap was recorded when the chromatid or chromosome with an unstained region existed or a cutting like a chromatid break was observed, and the width of the unstained part was clearly narrower than the chromatid and not dislocated from the axis. For numerical aberrations, the number of polyploid cells was counted by observing 200 cells per dose.

Clastogenic potential was assessed with the incidence of the total aberrant cells excluding cells with only gap. If the incidence of structural or numerical aberrations was less than 5%, it was judged to be negative (−). If the incidence of structural or numerical aberration in each treatment group was between 5% and 10% and reproducibility and/or dose-dependency was observed, it was judged to be inconclusive (±). If the incidence of structural or numerical aberration in each treatment group was 10% or more, and reproducibility and/or dose-dependency was observed, it was judged to be positive (+). These judgments were based on the methods of Ishidate (1987) and in-house background data.

28-Day toxicity study in rats

Animals and maintenance. Male and female SPF rats of Crj:CD(SD) strain (5 weeks old) were purchased from Japan Charles River, Inc. (Kanagawa, Japan). Animals were quarantined and acclimated to the testing facility for 9 days. Ten healthy animals of each sex were randomly assigned to four groups in an attempt to equalize mean group body weights by a randomized procedure. The rats were housed individually in aluminum cages with stainless steel wire mesh floors in an air-conditioned animal room (temperature, 23°C ± 3°C; relative humidity 55% ± 20%; lighting cycle, 12-hour light-dark). The rats were given commercial pelleted diet CRF-1 (Oriental Yeast Co., Ltd.) and tap water ad libitum through the acclimation and assay periods. The test substance, NHE-water, was administrated (20 mL/kg/day) to animals via intragastric infusion for 28 days. The control group received sterile water for injection (distilled water; DW).

Observations and measurements

Clinical observations, body weight and food consumption. All animals were observed for clinical signs and mortality twice a day (before dosing and 0.5–1 hour after dosing). Body weights were measured at the initiation of dosing, 8, 15, 22 and 28 days before administration of NHE-water, and the day of scheduled sacrifice. Food consumption was measured for each animal at the initiation of dosing, 8, 15, 22, and 28-day before administration of NHE-water.

Hematology

All animals were fasted from the evening on the day before necropsy, and blood samples obtained from the abdominal aorta under ether anesthesia at necropsy were collected into a bottle containing EDTA-K2. Hematocrit (Ht) values, hemoglobin (Hb) contents, red blood cell (RBC) counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (Plt) count, white blood cell (WBC) count, differential leukocyte counts, neutrophils (Neut), lymphocytes (Lym), monocytes (Mono), eosinophils (Eosi), basophils (Baso), large unstained cell (LUC) and reticulocyte (Reti) ratio were determined using a Hematology System (ADVIA 120; Bayer, Leverkusen, Germany).

Blood clotting test. Blood was collected into a bottle containing sodium citrate. The blood samples were centrifuged at 1700*g* for 13 min at the room temperature. The prothrombin time (PT), activated partial thromboplastin time (aPTT) and concentration of fibrinogen were measured using an automatically clot detection system STA compact (Roche, Basel, Switzerland).

Clinical chemistry. Clinical chemistry assessment was performed for all rats using blood samples collected from the abdominal aorta via a procedure similar to that used for blood collection for hematological assessment. The blood samples were centrifuged at 1700*g* for 7 min at the room temperature. The lactate dehydrogenase was examined using the plasma containing sodium heparin. Serum and plasma were used for examination of the following parameters using an automatic clinical chemistry analyzer Hitachi-7170 automatic Analyzer (Hitachi Ltd., Tokyo, Japan) or an automated electrolyte analyzer EA06R (A&T Co. Yokohama, Japan): total protein (TP), glucose (Glu), triglyceride (TG), total cholesterol (T.Cho.), phospholipid (PL), urea nitrogen (BUN), creatinine (Crea.), total bilirubin (T.Bil.), aspartate aminotransferase (AST), alanine aminotransferase (ALT),

γ-glutamyltranspeptidase (γ-GTP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), calcium (Ca), inorganic phosphorus (P), sodium (Na), potassium (K) and chloride (Cl).

Serum-protein electrophoresis test. A part of serum sample for clinical chemistry assessment was analyzed by a electrophoresis using an automatic electrophoresis system Epalyzer (Helena Kenkyujo K.K., Saitama, Japan) to determine the percentage of each protein and A:G ratio (A/G) in the blood by separating them into five distinct classes: albumin, alpha1-globulin (Alpha-1), alpha2-globulin (Alpha-2), beta-globulin (Beta) and gamma-globulins (Gamma). In addition, the concentration of each fraction was calculated by a ratio of each fraction versus the total protein value.

Urinalysis

Rats were placed in a metabolism cage and provided with both food and water. The urine samples were collected during 3-hour and 24-hour period from rats at day 28. As for the 3-hour period samples, urinary pH, occult blood, kentone bodies, glucose, protein, bilirubin and urobilinogen were measured using the test paper method (Multistix; Bayer Medical Ltd. Tokyo, Japan). As for the 24-hour period samples, urinary osmotic pressure was measured with an automatic osmometer, Osmotic Pressure AUTO&STAT OM-6030 (Arkray Factory, Inc. Shiga, Japan). The urine samples were centrifuged at 420*g* for 5 min and analyzed sodium (Na), potassium (K) and chloride (Cl) using an automated electrolyte analyzer EA06R, and urine sediment was checked by modified Sternheimer stain under microscopy.

Ophthalmological examinations

Eyes were examined during prior to study termination using an indirect ophthalmoscope. Animals with ocular abnormalities of external appearance of the eyes, the anterior part, the vitreous body and the fundus oculi of the eyes were examined after the application of the mydriatic agent, Midorin P (Santen, Osaka, Japan).

Pathology and histopathology

Following euthanasia by exsanguination on day 29, all animals were necropsied and gross observations were recorded. Organ weights were recorded for the heart, lungs, liver, kidneys, spleen, adrenals, testes, ovaries, thyroid glands, pituitary gland, thymus, prostate, uterus, salivary glands, seminal vesicles and brain. Eyes were placed in Davidson's solution and then transferred to 10% formalin. Testes were placed in formalin-sucrose-acetic (FSA) solution and then transferred to 10% neutral buffered formalin. All other tissues and carcasses were preserved in 10% neutral buffered formalin. After paraffin embedding, the excised organs and tissues were cut at 3 to 4 μm; the sections were placed on glass slides and stained with hematoxylin and eosin.

Statistical analysis

Data were analyzed for homogeneity of variance using Bartlett's test. Homogenous data were analyzed using the parametric one-way analysis of variance (ANOVA), and the significance of differences was assessed using the Dunnet's multiple comparison test to compare the values between the control group and a NHE-water-administered group. Heterogeneous data were analyzed using the Steel's test. Treatment differences were assessed at a 5% level of significance ($p < 0.05$).

Results

Bacterial reverse mutation test

The mutagenicity test was conducted at five concentrations (6.25% to 100%). As shown in Table 1, the number of revertants in a main study did not increase to more than twice versus that of the negative control in any strains used, in either presence or absence of S9 mix. NHE-water did not inhibit growth of any bacterial strains at any concentrations up to the limit dose of 100% concentration with or without S9 mix. Moreover, no precipitation of the test substance was observed in any bacterial strains. No microbial contamination was detected in the test solution or S9 mix in any of the tests. The mutagenicity of the positive-control substances confirmed the sensitivity of the test and the activity of the S9 mix, and the numbers of revertants in the positive and negative controls were within the range of historical control values (data not shown). The data of the test were judged as acceptable because (1) the negative-control values were appropriate in comparison with the background values; (2) the positive-control value was more than twice versus the negative-control value, and appropriate in

Table 1. Results of bacterial reverse mutation test of NHE-water[a]

		Mean number of revertants (number of coloniesies/plate)				
		Base-pair substitution type			Frameshift type	
	Test substance concentration (%)	TA100	TA1535	WP2uvrA	TA98	TA1537
S9 mix (–)	0	115	15	9	27	20
	6.25	122	11	10	25	20
	12.5	111	14	11	23	22
	25	122	11	6	20	25
	50	102	17	9	26	22
	100	123	14	5	22	23
S9 mix (+)	0	108	11	24	24	16
	6.25	108	8	21	20	15
	12.5	132	13	21	31	18
	25	109	11	26	28	15
	50	119	10	21	31	17
	100	120	11	22	27	12
Positive control S9 mix (-)	Chemical	AF2	NaN_3	AF2	AF2	9-AA
	Dose (μg/plate)	0.01	0.5	0.01	0.1	80
	Mean revertant colonies/plate	743	558	140	693	225
Positive control S9 mix (–)	Chemical	2-AA	2-AA	2-AA	2-AA	2-AA
	Dose (μg/plate)	1	2	10	0.5	2
	Mean revertant colonies/plate	1491	459	486	454	185

[a] Sterile water for injection (the negative control). See materials and methods for abbreviations.

comparison with the background values and (3) there were no abnormalities in the sterility test.

Chromosomal aberration test in CHL/IU cells

Marked cell growth inhibition was not observed at the highest doses in all treatments. In all of the three treatment scenarios, the incidences of cells with chromosome structural aberrations and numerical aberrations in the groups treated with NHE-water were similar to those in the negative-control group (Tables 2–4). No precipitation of the test substance was observed in all treatments. In cultures treated with the positive controls, high incidences of chromosomal aberrations were observed, confirming the sensitivity of the test and the activity of the S9 mix. The incidence of cells with chromosome aberrations in both the negative- and positive-control groups was within the range of the historical data (data not shown), which supported the validity of the present study.

28-Day toxicity study in rats

No death and no clinical signs related to the administration of NHE-water were observed during the administration period. There were no significant differences related to the administration of NHE-water during the administration period, although the body weight gain of the NHE-water-administrated rats had a slight tendency to increase than that of the control group in both sexes (Figure 2). There were no significant differences related to the administration of NHE-water during the administration period, although the cumulative food consumption of the NHE-water-administrated rats had a tendency to increase as compared with the control group (Figure 3).

The results of hematology and blood clotting test for male and female rats administrated with NHE-water or water are presented in Tables 5 and 6. Although a significant change of the rate of basophils ($p < 0.05$) was observed in females in the NHE-water group (the control group, 0.1% ± 1%; the NHE-water group, 0.2% ± 1%), this change was

Table 2. Chromosome analysis of Chinese hamster cells (CHL/IU) treated with NHE-water: short-term treatment (-S9)

Group	Concentration (%)	S9 mix	T ime of exposure (h)	Relative cell growth (%)	Number of Cells analyzed	Number of cells with structural aberrations						Number of cells with aberrations except gap (%)	Number of cells analyzed for polyploid	Number of poly-ploidy cells (%)	Final judgement
						gap	ctb	cte	csb	cse	oth				
NHE-water	0[a]	–	6	100	200	3	0	0	0	0	0	0 (0.0)	200	1 (0.5)	–
	25	–	6	80.0	200	1	2	0	0	0	0	2 (1.0)	200	0 (0.0)	–
	50	–	6	94.5	200	1	0	0	0	0	0	0 (0.0)	200	0 (0.0)	–
	100	–	6	93.4	200	3	0	0	0	1	0	1 (0.5)	200	3 (1.5)	–
MMC (μg/mL)	0.1[b]	–	6	78.6	200	12	20	54	0	0	0	67 (33.5)	200	0 (0.0)	+

[a] Negative control (sterile water for injection).
[b] Positive control (MMC; mitomycin C). See materials and methods for abbreviations.

Table 3. Chromosome analysis of Chinese hamster cells (CHL/IU) treated with NHE-water: short-term treatment (+S9)

Group	Concentration (%)	S9 mix	T ime of exposure (hours)	Relative cell growth (%)	Number of Cells analyzed	Number of cells with structural aberrations						Number of cells with aberrations except gap (%)	Number of cells analyzed for polyploid	Number of poly-ploidy cells (%)	Final judgement
						gap	ctb	cte	csb	cse	oth				
NHE-water	0[a]	+	6	100	200	2	0	1	0	0	0	1 (0.5)	200	0 (0.0)	–
	25	+	6	109.4	200	1	0	0	0	0	0	2 (1.0)	200	1 (0.5)	–
	50	+	6	88.8	200	3	0	1	0	0	0	1 (0.5)	200	2 (1.0)	–
	100	+	6	89.3	200	0	0	1	0	0	0	1 (0.5)	200	1 (0.5)	–
CP (μg/mL)	12.5[b]	+	6	97.0	200	5	7	25	0	0	0	30 (15.0)	200	0 (0.0)	+

[a] Negative control (sterile water for injection).
[b] Positive control (CP; cyclophosphamide). See materials and methods for abbreviations.

Table 4. Chromosome analysis of Chinese hamster cells

Group	Concentration (%)	S9 mix	Time of exposure (hours)	Relative cell growth (%)	Number of Cells analyzed	Number of cells with structural aberrations						Number of cells with aberrations except gap (%)	Number of cells analyzed for polyploid	Number of polyploidy cells (%)	Final judgement
						gap	ctb	cte	csb	cse	oth				
NHE-water	0[a]	–	24	100.0	200	2	0	0	0	0	0	0 (0.0)	200	1 (0.5)	–
	25	–	24	102.4	200	0	0	0	0	0	0	0 0.0)	200	0 (0.0)	–
	50	–	24	104.8	200	0	0	0	0	1	0	1 (0.5)	200	0 (0.0)	–
	100	–	24	101.6	200	2	0	0	0	0	0	0 (0.0)	200	0 (0.0)	–
MMC (μg/mL)	0.05[b]	–	24	109.3	200	5	13	3	37	0	0	46 (23.0)	200	0 (0.0)	+

[a] Negative control (sterile water for injection).
[b] Positive control (MMC; mitomycin C). See materials and methods for abbreviations.

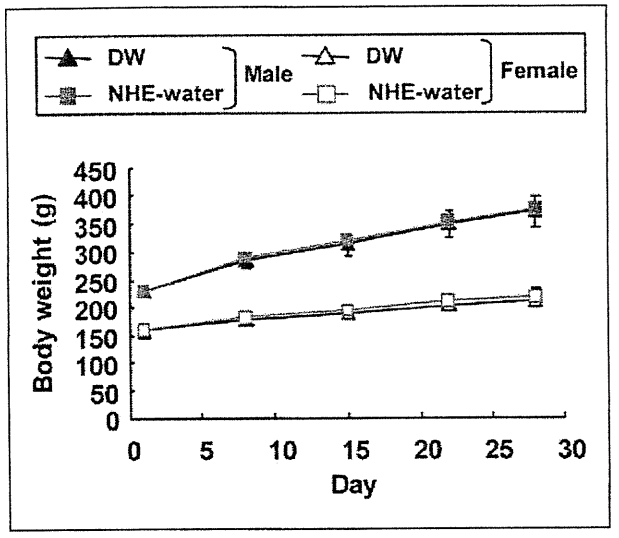

Figure 2. Body weight curves for males ($n = 10$) and females ($n = 10$) rats during a 28-day study. The values are expressed as mean ± SD.

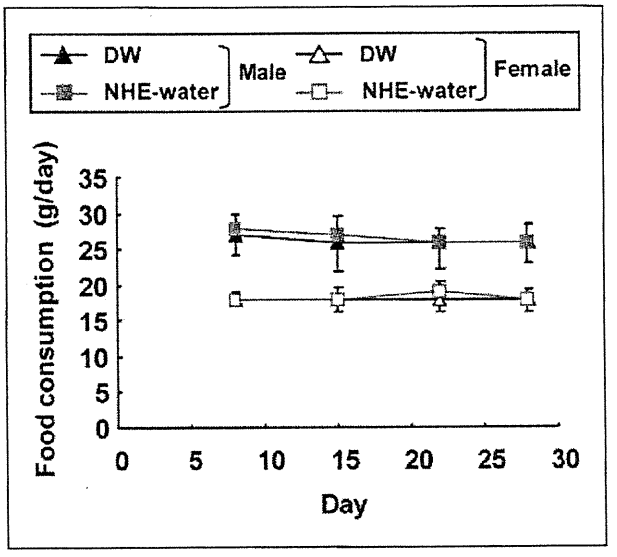

Figure 3. Food consumption curves for males ($n = 10$) and females ($n = 10$) rats during a 28-day study. The values are expressed as mean ± SD.

Table 5. Hematological evaluation in rats treated orally with DW or NHE-water for 28 days[a]

	DW		NHE-water	
	Male	Female	Male	Female
Ht (%)	45.7 ± 1.2	45.1 ± 1.5	46.1 ± 1.4	45.2 ± 1.0
Hb (g/dL)	15.3 ± 0.4	15.5 ± 0.7	15.4 ± 0.6	15.5 ± 0.5
RBC(10^5/μL)	8.24 ± 0.31	8.16 ± 0.22	8.26 ± 0.39	8.19 ± 0.13
MCV p)	55.5 ± 2.7	55.2 ± 0.9	55.8 ± 2.1	55.2 ± 0.9
MCH (pg)	18.6 ± 0.9	19.0 ± 0.5	18.7 ± 0.6	18.9 ± 0.5
MCHC (%)	33.5 ± 0.4	34.4 ± 0.6	33.5 ± 0.5	34.3 ± 0.5
Plt (10^3/μL)	1057 ± 110	1159 ± 66	1026 ± 73	1156 ± 81
WBC (10^3/μL)	10.01 ± 1.79	7.36 ± 2.93	10.52 ± 1.95	9.38 ± 2.31
Differential leukocyte counts				
Neut	14.5 ± 4.6	15.0 ± 5.7	15.0 ± 5.7	14.3 ± 4.3
Lym (%)	81.2 ± 4.6	81.2 ± 5.4	80.7 ± 3.9	81.6 ± 4.7
Mono (%)	2.4 ± 0.5	1.8 ± 0.7	2.3 ± 1.0	2.0 ± 0.7
Eosi (%)	1.0 ± 0.3	1.2 ± 0.3	1.0 ± 0.5	1.1 ± 0.3
Baso (%)	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1[b]
LUC (%)	0.8 ± 0.2	0.8 ± 0.3	0.9 ± 0.2	0.9 ± 0.2
Reti (%)	2.3 ± 0.3	1.8 ± 0.4	2.3 ± 0.2	2.0 ± 0.9

[a] Values shown as mean ± SD. See materials and methods for abbreviations
[b] Significant difference from control group, $p < .05$.

not considered to be related to the administration with NHE-water, because it was within a normal range. No other significant changes were noted in both hematology and blood clotting test parameters.

The results of clinical chemistry and serum protein electrophoresis test for male and female rats administered with NHE-water or sterile water are presented in Tables 7 and 8. Although significant changes of the AST and ALT values ($p < 0.05$) were observed in males in the NHE-water group (AST; the control group, 75 ± 6 U/L; the NHE-water group, 69 ± 5 U/L, ALT; the control group, 29 ± 2 U/L; the NHE-water group, 28 ± 2 U/L), these changes were not considered to be related to the NHE-water

Table 6. Blood clotting test in rats treated orally with DW or NHE-water for 28 days[a]

	DW		NHE-water	
	Male	Female	Male	Female
PT (sec)	18.9 ± 1.9	15.5 ± 0.7	17.8 ± 1.9	15.3 ± 0.4
aPTT (sec)	27.1 ± 2.7	20.5 ± 2.1	26.6 ± 2.5	21.4 ± 1.8
Fibrinogen (mg/dL)	311 ± 24	250 ± 28	315 ± 18	249 ± 32

[a] Values shown as mean ± SD. See materials and methods for abbreviations.

Table 7. Clinical chemistry in rats treated orally with DW or NHE-water for 28 days[a]

	DW		NHE-water	
	Male	Female	Male	Female
TP (g/dL)	5.69 ± 0.29	6.07 ± 0.39	5.68 ± 0.23	6.06 ± 0.28
Glu. (mg/dL)	162 ± 15	128 ± 19	155 ± 14	133 ± 18
TG (mg/dL)	43.7 ± 15.7	21.2 ± 21.5	45.1 ± 21.9	20.5 ± 9.3
T. Cho. (mg/dL)	63 ± 6	80 ± 11	65 ± 10	80 ± 14
PL (mg/dL)	102 ± 8	137 ± 23	105 ± 14	137 ± 25
BUN (mg/dL)	11.2 ± 1.3	15.1 ± 2.8	11.9 ± 0.9	14.9 ± 1.6
Crea. (mg/dL)	0.23 ± 0.03	0.26 ± 0.04	0.23 ± 0.02	0.27 ± 0.03
T. Bil. (mg/dL)	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.04 ± 0.03
AST (U/L)	75 ± 6	69 ± 11	69 ± 5[b]	71 ± 11
ALT (U/L)	29 ± 2	24 ± 5	28 ± 2[b]	24 ± 5
GTP (U/L)	0.5 ± 0.2	0.7 ± 0.2	0.4 ± 0.2	0.9 ± 0.3
ALP (U/L)	695 ± 122	388 ± 85	640 ± 64	393 ± 72
LDH (U/L)	347 ± 129	330 ± 113	321 ± 112	314 ± 95
CPK (U/L)	184 ± 41	144 ± 40	190 ± 56	146 ± 42
Ca (mg/dL)	9.92 ± 0.32	9.95 ± 0.25	9.85 ± 0.35	9.87 ± 0.28
P (mg/dL)	8.02 ± 0.33	7.60 ± 1.25	8.07 ± 0.75	7.22 ± 0.81
Na (mmol/L)	144.4 ± 1.2	143.3 ± 2.2	144.1 ± 0.7	142.1 ± 1.1
K (mmol/L)	4.39 ± 0.32	4.46 ± 0.41	4.49 ± 0.40	4.22 ± 0.22
Cl (mmol/L)	108.3 ± 1.2	109.7 ± 2.1	109.0 ± 1.3	108.9 ± 1.4

[a] Values shown as mean ± SD. See Materials and Methods for abbreviations.
[b] Significant difference from control group, $p<.05$

Table 8. Serum protein electrophoresis test in rats treated orally with DW or NHE-water for 28 days[a]

	DW		NHE-water	
	Male	Female	Male	Female
Albumin (g/dL)	2.80 ± 0.17	3.21 ± 0.27	2.80 ± 0.11	3.15 ± 0.18
Alpha-1 (g/dL)	1.24 ± 0.15	1.12 ± 0.13	1.21 ± 0.13	1.16 ± 0.12
Alpha-2 (g/dL)	0.48 ± 0.05	0.51 ± 0.04	0.51 ± 0.04	0.52 ± 0.03
Beta (g/dL)	0.86 ± 0.06	0.89 ± 0.05	0.87 ± 0.07	0.87 ± 0.05
Gamma (g/dL)	0.31 ± 0.04	0.35 ± 0.06	0.30 ± 0.07	0.36 ± 0.08

[a] Values shown as mean ± SD. See materials and methods for abbreviations.

Table 9. Summary of organ weight/body weight ratio (% of body weight)[a]

	DW		NHE-water	
	Male	Female	Male	Female
Heart	0.361 ± 0.015	0.382 ± 0.021	0.361 ± 0.035	0.388 ± 0.016
Lungs	0.346 ± 0.031	0.442 ± 0.028	0.359 ± 0.026	0.455 ± 0.026
Liver	3.091 ± 0.296	3.022 ± 0.118	2.925 ± 0.153	3.012 ± 0.185
Kidneys	0.776 ± 0.068	0.832 ± 0.086	0.765 ± 0.037	0.812 ± 0.045
Spleen	0.186 ± 0.021	0.192 ± 0.024	0.179 ± 0.021	0.220 ± 0.038
Adrenals	0.018 ± 0.002	0.033 ± 0.003	0.017 ± 0.002	0.032 ± 0.005
Testes	0.941 ± 0.081	–	0.916 ± 0.060	–
Ovaries	–	0.039 ± 0.008	–	0.037 ± 0.007
Thyroid glands	0.007 ± 0.001	0.008 ± 0.002	0.007 ± 0.001	0.008 ± 0.001
Pituitary gland	0.004 ± 0.001	0.006 ± 0.001	0.003 ± 0.000	0.006 ± 0.001
Thymus	0.129 ± 0.027	0.209 ± 0.027	0.138 ± 0.038	0.216 ± 0.054
Prostate	0.289 ± 0.047	–	0.298 ± 0.038	–
Uterus	–	0.231 ± 0.118	–	0.231 ± 0.080
Salivary glands	0.174 ± 0.009	0.202 ± 0.020	0.180 ± 0.020	0.187 ± 0.020
Seminal vesicles	0.312 ± 0.042	–	0.300 ± 0.052	–
Brain	0.585 ± 0.044	0.937 ± 0.061	0.569 ± 0.043	0.913 ± 0.056

[a] Values shown as mean ± SD.

administration, because it was within a normal range. No other significant changes were noted in both clinical chemistry and serum protein electrophoresis test parameters.

There were no differences in urinalysis and ophthalmological examinations between treated and control animals in both sexes. No abnormalities were observed in any treated or control animals at necropsy.

Although a significant increase in the absolute weight of the spleen was observed in the females in the NHE-water group, the weight ratio of spleen versus body was not changed (Table 9). There were no significant treatment-related changes in the absolute weight and body weight ratio in other organs. Histopathological examination of tissues from the control and NHE-water group revealed no treatment-related differences.

Discussion

To provide evidences of the safety of NHE-water, we evaluated the mutagenicity, genotoxicity and subchronic oral toxicity of NHE-water in the present study. The test substance, NHE-water, was evaluated to be negative in the bacterial reverse mutation, chromosomal aberration and rat 28-day toxicity study.

The results of these assays demonstrate that NHE-water (1) does not induce reverse mutations in *S. typhimurium* and *E. coli* at concentrations as high as 100% dose/plate (Table 1); (2) does not induce chromosomal aberration in cultured CHL/IU cells exposed to concentrations up to 100% dose/plate (Tables 2–4) and (3) is devoid of adverse effects when orally administered to rats at doses of 20 mL/kg/day (Figures 2 and 3, and Tables 5–9).

In the rat 28-day toxicity study, a few statistically significant changes in hematology (i.e., basophils ratio in female rats) and clinical chemistry parameters (i.e., decreased AST and decreased ALT in male rats) were noted (Table 7). These changes were not considered biologically significant because the differences were negligible, occurred only in one sex and restored within the normal range. The only significant increase in the absolute weight of the spleen was observed in the females in the NHE-water group, but the weight ratio of spleen versus body was not changed (Table 9), and no significant changes were found in both necropsy and histopathological examination. From these results, the changes were judged to be incidental and were not considered to be of toxicological significance.

Because no toxic changes were observed at 20 mL/kg/day in both sexes, the no observed adverse effect level (NOAEL) for NHE-water was estimated to be more than 20 mL/kg/day under the present study conditions. Specifically, these data are consistent with the

expected safety of human consumption of NHE-water up to at least 1.2 L/day in individuals with a normal body weight of 60 kg, determined as follows: 20 mL/kg/day × 60 kg = 1200 mL/day.

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Environmental and Molecular Mutagenesis 52:244–252 (2011)

Research Article

Antigenotoxic Effects of *p53* on Spontaneous and Ultraviolet Light B–Induced Deletions in the Epidermis of *gpt* Delta Transgenic Mice

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Tumor development in the skin may be a multistep process where multiple genetic alterations occur successively. The *p53* gene is involved in genome stability and thus is referred to as "the guardian of the genome." To better understand the antigenotoxic effects of *p53* in ultraviolet light B (UVB)-induced mutagenesis, mutations were measured in the epidermis of UVB-irradiated $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice. In the mouse model, point mutations and deletions are separately identified by the *gpt* and Spi$^-$ assays, respectively. The mice were exposed to UVB at single doses of 0.5, 1.0, or 2.0 kJ/m^2. The mutant frequencies (MFs) were determined 4 weeks after the irradiation. All doses of UVB irradiation enhanced *gpt* MFs by about 10 times than that of unirradiated mice. There were no significant differences in *gpt* MFs and the mutation spectra between $p53^{+/+}$ and $p53^{-/-}$ mice. The predominant mutations induced by UVB irradiation were G:C to A:T transitions at dipyrimidines. In contrast, in unirradiated $p53^{-/-}$ mice, the frequencies of Spi$^-$ large deletions of more than 1 kb and complex-type deletions with rearrangements were significantly higher than those of the Spi$^-$ large deletions in $p53^{+/+}$ counterparts. The specific Spi$^-$ mutation frequency of more than 1 kb deletions and complex types increased in a dose-dependent manner in the $p53^{+/+}$ mice. However, no increase of such large deletions was observed in irradiated $p53^{-/-}$ mice. These results suggest that the antigenotoxic effects of *p53* may be specific to deletions and complex-type mutations induced by double-strand breaks in DNA. Environ. Mol. Mutagen. 52:244–252, 2011.

Key words: Spi; deletion mutations; dipyrimidines; mutation frequency

INTRODUCTION

The *p53* gene is involved in various aspects of genome stability and thus is referred to as "the guardian of the genome." Several strains of transgenic mice were developed in which a major part of *p53* was deleted [Donehower et al., 1992; Tsukada et al., 1993; Jacks et al., 1994; Purdie et al., 1994], and each of these knockout strains has a propensity to develop thymic lymphoma at an early stage. However, no significant difference in spontaneous *lacI* mutant frequencies (MFs) has been reported between $p53^{+/+}$ and $p53^{-/-}$ mice [Nishino et al., 1995; Buettner et al., 1996]. In addition, *lacI* MFs induced after exposure to 4-nitroquinoline 1-oxide (4-NQO) are not significantly different between $p53^{+/+}$ and $p53^{-/-}$ mice [Sands et al., 1995]. Because the *lacI* transgene preferentially detects point mutations [Nishino et al., 1995], it was suggested that *p53* deficiency did not affect accumulation of

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point mutations in vivo. However, it remains uncertain, whether *p53* deficiency affects other types of mutations or chromosome aberrations. In fact, a study with the endogenous *aprt* gene, which detects loss of heterozygosity (LOH) induced by chromosome aberrations and point mutations, showed that frequencies of chromosome aberrations were significantly increased in $p53^{-/-}$ mice compared with $p53^{+/+}$ [Shao et al., 2000]. Another study, using the endogenous p^{un} reversion mutation assay, suggested that *p53* was involved in X-ray-induced intrachromosomal recombination [Aubrecht et al., 1999]. Loss of *p53* function was associated with an increase in the frequency of LOH within the *tk* gene in TK6 human cells in vitro [Morris, 2002]. These studies suggest that the effect of *p53* loss may be influential in deletions and/or rearrangement-type mutations induced by DNA double-strand breaks (DSB).

To further investigate the effects of *p53* on point mutations and deletions in vivo, we analyzed mutations in the epidermis of $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice exposed to ultraviolet light B (UVB) irradiation. In this mouse model, point mutations and deletions are separately identified by the *gpt* and Spi$^-$ assays [Nohmi et al., 1996, 2000; Nohmi, 2007]. UVB is responsible for most carcinogenic effects of sun exposure. The sunlight-induced mutation spectrum is highly UV-specific, quite similar to that induced by UVB [Ikehata et al., 2004]. UVB irradiation induces not only point mutations, such as G:C to A:T transitions, but also deletions of more than 1 kb in the epidermis of *gpt* delta mice [Horiguchi et al., 1999, 2001]. In this study, we demonstrated that Spi$^-$ MFs in unirradiated $p53^{-/-}$ mice were significantly higher than that in $p53^{+/+}$ counterparts. In the $p53^{-/-}$ background, large deletions greater than 1 kb in size and complex-type rearrangements were much more frequently observed, compared with the $p53^{+/+}$ counterparts. Interestingly, the specific Spi$^-$ mutation frequency of the large deletions and complex types increased in a dose-dependent manner in the $p53^{+/+}$ mice. However, no increase in frequencies of such large deletions was observed in irradiated $p53^{-/-}$ mice. There were no significant differences in the *gpt* MF and spectra between $p53^{+/+}$ and $p53^{-/-}$ mice regardless of the presence or the absence of UVB irradiation. The results suggest that *p53* affects the induction of deletions and rearrangements but not the induction or accumulation of point mutations in vivo.

MATERIALS AND METHODS

UVB Irradiation of $p53^{+/+}$ and $p53^{-/-}$ gpt Delta Mice

Homozygous *gpt* delta transgenic mice of C57BL/6J genetic background [Nohmi et al., 2000] were mated with $p53^{-/-}$ mice of C57BL/6J background [Tsukada et al., 1993], and $p53^{+/-}$ *gpt* delta mice were obtained. We obtained $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice by intercrossing $p53^{+/-}$ *gpt* delta mice. Genotypes of the *p53* and *gpt* genes were examined by polymerase chain reaction (PCR) as described in the study of Tsukada et al. (1993), Nohmi et al. (1996). In this study, male $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice (6–10 weeks old) were used. The experimental protocol described in the following sections was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. For UVB irradiation of the skin, the hair on the back was removed using a clipper and hair removing cream under anesthesia. Four days later, the mice were exposed to UVB at a single dose of 0.5, 1.0, or 2.0 kJ/m^2 (0.3 kJ/m^2/min) under anesthesia. The UV source was a model UVM-57 (UVP, Upland, CA). Dose rate was measured by a UVX-31 Radiometer (UVP, Upland, CA). Irradiation was performed in a CHROMATO-VUE® CABINET model CC-10 (UVP, Upland, CA). Four mice were used in each group. They were sacrificed 4 weeks later and the back skin was collected and stored at -80°C. The epidermis was separated from the skin using thermolysin treatment and genomic DNA was extracted by the phenol/chloroform/isoamyl alcohol method [Ikehata et al., 2001]. Lambda EG10 phages were rescued from the genomic DNA by in vitro packaging reactions using Transpack® packaging extract (Stratagene, La Jolla, CA) and used for the following mutation assays.

Mutation Assay and Sequencing Analysis

The *gpt* mutation assay was performed as described previously [Nohmi et al., 2000]. The rescued phages were used to infect *E. coli* strain YG6020 expressing Cre recombinase to convert the transgene to plasmid. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol (Cm) and 6-thioguanine (6-TG). The plates were incubated for 3 days at 37°C to select for colonies harboring the plasmid carrying the mutated *gpt* gene. Infected cells were also poured on plates containing Cm without 6-TG to determine the number of rescued plasmids. The *gpt* MF was calculated as described previously [Nohmi et al., 2000]. The selected 6-TG–resistant mutants were cultured and collected. A 739-bp DNA fragment containing the mutated *gpt* gene was amplified by PCR [Nohmi et al., 2000]. DNA sequencing of the *gpt* gene was performed with BigDye® Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA) on a ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequencing primer was the gptA2 primer (5′-TCTCGCGCAACCTATTTTCCC-3′). Clonally corrected mutation spectra were estimated, that is, counting the same mutation from the same animal as one mutation.

The Spi$^-$ mutation assay was performed as described previously [Nohmi et al., 2000] with some modifications. We added 10 mM $MgSO_4$ to both agar plates and soft agar to improve the detection efficiency of Spi$^-$ plaques, as described previously [Shibata et al., 2003]. The rescued phages from the genomic DNA of $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice were used to infect *E. coli* XL1-Blue MRA (P2). Infected cells were mixed with molten soft agar, poured onto lambda-trypticase agar plates, and incubated at 37°C. The plaques detected on the plates (Spi$^-$ candidates) were suspended in 50 μl of SM buffer. The suspension was spotted on two types of plates, spread with either XL1-Blue MRA (P2) or the WL95 (P2) strain. The plates were incubated overnight at 37°C. The number of mutants that made clear spots on both strains was counted as confirmed Spi$^-$ mutants. The Spi$^-$ MF was calculated as described previously [Nohmi et al., 2000]. The MFs were corrected for potential clonal expansion. The lysates of the Spi$^-$ mutants were obtained by infection of *E. coli* LE392 with the recovered Spi$^-$ mutants. The lambda DNA was extracted from the lysates with the Quantum Prep® AquaPure™ Genomic DNA Isolation Kit (BIO-RAD, Hercules, CA). The lysate and extracted DNA were used as templates for PCR analysis to determine the deleted regions. The DNA fragments containing the deletions which were amplified by PCR using primers 001–002 (5 kb in length), 005–012 (14 kb), or 005–006 (21 kb), followed by sequencing analysis of the PCR products. The PCR primers were:

- primer 001 (5′-CTCTCCTTTGATGCGAATGCCAGC-3′),
- primer 002 (5′-GGAGTAATTATGCGGAACAGAATCATGC-3′),

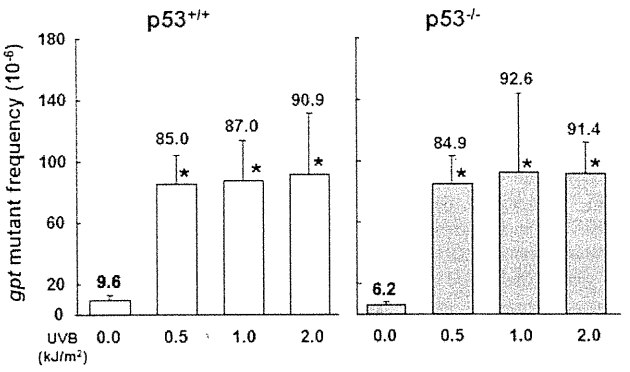

Fig. 1. *gpt* MFs in the epidermis of UVB-irradiated $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice. * Denotes $P < 0.05$ ($n = 4$) in Dunnett test (for $p53^{+/+}$) or Kruskal–Wallis test (for $p53^{-/-}$) comparing the MFs of UVB-irradiated versus the corresponding unirradiated mice. Vertical bars show the standard deviations with mice as the unit of comparison.

- primer 005 (5′-CGTGGTCTGAGTGTGTTACAGAGG-3′),
- primer 006 (5′-GTTATGCGTTGTTCCATACAACCTCC-3′), and
- primer 012 (5′-CGGTCGAGGGACCTAATAACTTCG-3′).

Sequence changes within and outside of the *gam/redBA* genes were identified by DNA sequencing analysis. The appropriate primers for DNA sequencing were selected based on the results of PCR analysis. The entire sequence of lambda EG10 is available at http://dgm2alpha.nihs.go.jp.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analyses were performed by analysis of variance followed by parametric (Dunnett) or nonparametric (Kruskal–Wallis) test to compare between two groups. Cochran–Armitage trend test was applied for significance of dose-dependent increase as noted in a detailed review of transgenic rodent mutation assays [Lambert et al., 2005]. A comparison of mutational spectra was performed using Adams–Skopek test [Adams and Skopek, 1987; Cariello et al., 1994]. A P value less than 0.05 denoted a statistically significant difference.

RESULTS

Increased *gpt* MFs in the Epidermis of UVB-Irradiated $p53^{+/+}$ and $p53^{-/-}$ Mice

The *gpt* MFs in the epidermis of $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice were significantly increased by UVB irradiation at 0.5, 1.0, or 2.0 kJ/m^2 compared with unirradiated mice (Fig. 1). In $p53^{+/+}$ mice, *gpt* MFs of the irradiated groups were 85.0–90.9 × 10^{-6}, which were similar to those of the irradiated groups of the $p53^{-/-}$ mice (84.9–92.6 × 10^{-6}). These values were 9–15 times higher than those of unirradiated mice (9.6 × 10^{-6} and 6.2 × 10^{-6}, respectively, for $p53^{+/+}$ and $p53^{-/-}$ mice). The *gpt* MFs in the unirradiated groups were not significantly different between $p53^{+/+}$ and $p53^{-/-}$ mice. There were no dose-dependent increases in *gpt* MFs regardless of the *p53* status. This may suggest that *gpt* MFs in the epidermis were saturated at UVB doses greater than 0.5 kJ/m^2.

Induction of G:C to A:T Transitions at Dipyrimidines in the Epidermis of UVB-Irradiated $p53^{+/+}$ and $p53^{-/-}$ Mice

To investigate the type of mutations induced by UVB irradiation, we determined the *gpt* mutation spectra in the epidermis of $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice (Tables I and II). In UVB-irradiated $p53^{+/+}$ mice, G:C to A:T transitions were the predominant mutations, i.e., 78% (28/36), 76% (22/29), and 74% (28/38) at 0.5, 1.0, and 2.0 kJ/m^2, respectively. Most of the G:C to A:T transitions were observed on the 3′ side of the dipyrimidines that were 5′-C<u>C</u>-3′ and 5′-T<u>C</u>-3′, that is, 93% (26/28), 95% (21/22), and 86% (24/28) of G:C to A:T transitions for 0.5, 1.0, and 2.0 kJ/m^2, respectively. No significant differences in the *gpt* mutation spectra were observed between UVB-irradiated groups. In UVB-irradiated $p53^{-/-}$ mice, G:C to A:T transitions were 65% (22/34), 81% (29/36), and 69% (20/28) of mutations for 0.5, 1.0, and 2.0 kJ/m^2, respectively. The G:C to A:T transitions observed on the 3′ side of the dipyrimidines, 5′-C<u>C</u>-3′ and 5′-T<u>C</u>-3′, were 91% (20/22), 83% (24/29), and 95% (19/20) of G:C to A:T transitions for 0.5, 1.0, and 2.0 kJ/m^2, respectively. The tandem base substitution, 5′-<u>CC</u>-3′ to 5′-<u>TT</u>-3′, was observed in $p53^{+/+}$ and $p53^{-/-}$ mice. The characteristics of the *gpt* mutation spectra were similar between irradiated $p53^{+/+}$ and $p53^{-/-}$ mice ($P = 0.91$, Adams–Skopek test). In untreated mice, base substitutions such as G:C to A:T, G:C to T:A, and A:T to T:A were observed in both $p53^{+/+}$ (86%, 32/37) and $p53^{-/-}$ (89%, 16/18) mice.

High-Spontaneous Spi$^-$ MFs in $p53^{-/-}$ Mice

To investigate the induction of deletions, Spi$^-$ MFs in the epidermis of $p53^{+/+}$ and $p53^{-/-}$ *gpt* delta mice were determined. In $p53^{+/+}$ mice, Spi$^-$ MFs were significantly increased by UVB irradiation at 1.0 or 2.0 kJ/m^2 compared with those of unirradiated mice (Table III). The Spi$^-$ MFs were increased four times for the 1.0 and 2.0 kJ/m^2 doses compared with the control level. In $p53^{-/-}$ mice, the Spi$^-$ MF of unirradiated mice was 4.91 × 10^{-6}, which was 2.5 times higher than that of $p53^{+/+}$ mice (1.96 × 10^{-6}). In the UVB-irradiated groups, Spi$^-$ MFs were 1.2- to 1.7-fold higher but not significantly different from those of the unirradiated mice.

Characteristics of Spi$^-$ Large Deletions in $p53^{+/+}$ and $p53^{-/-}$ Mice

To characterize the UVB-induced deletion mutations in more detail, the Spi$^-$ mutants rescued from the epidermis were subjected to PCR analysis. The Spi$^-$ mutants that had the deletions with the sizes more than 1 kb were selected and analyzed by DNA sequencing. The specific