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

Yasukazu Saitoh<sup>1</sup>, Yoshikazu Harata<sup>2</sup>,  
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 Nobuhiko Miwa<sup>1</sup>

## 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 peroxyntrites (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, Shiohinden, 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

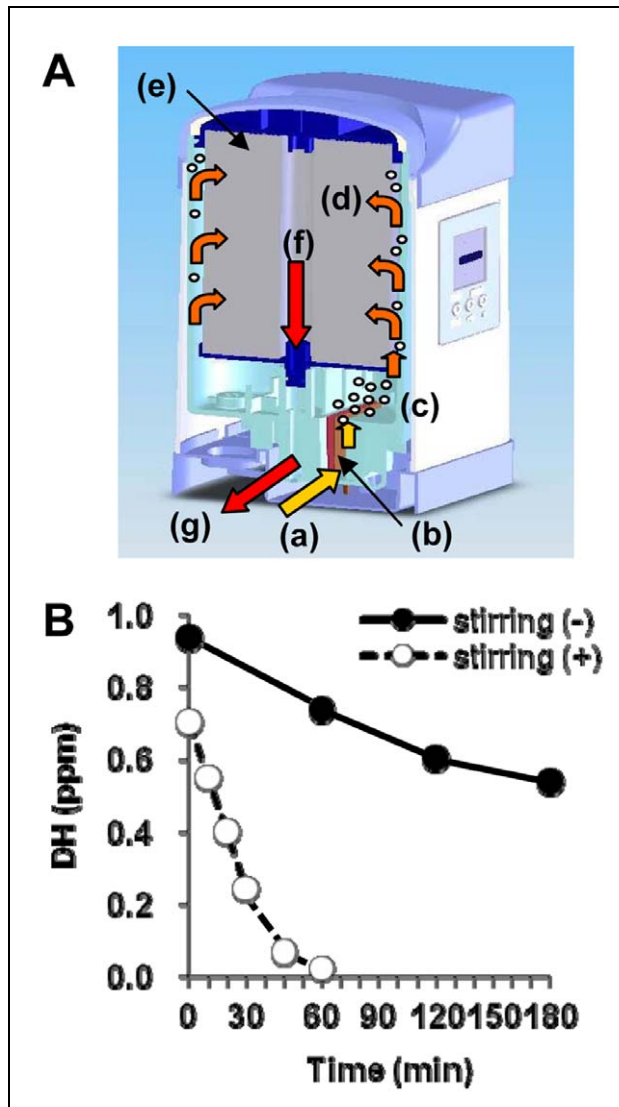
and Management of Animals, 1982)], 'Standards Relating to the Care and Management of Experimental Animals' [Notice No. 6 of the Prime Minister's office, 27 March 1980: hereinafter referred to as 'the experimental animal standards' (Standards Relating to the Care and Management of Experimental Animals, 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<sub>3</sub>), 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  $\mu\text{g}/\text{mL}$  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  $\mu\text{g}/\text{mL}$ .

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,  $\text{MgCl}_2$  8  $\mu\text{mol}$ , KCl 33  $\mu\text{mol}$ , glucose-6-phosphate (G-6-P) 5  $\mu\text{mol}$ , NADPH 4  $\mu\text{mol}$ , NADH 4  $\mu\text{mol}$  and sodium phosphate buffer (pH 7.4) 100  $\mu\text{mol}$  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  $\text{NaN}_3$ ; a vehicle control (sterile water for injection) was also included. Tests were performed by the preincubation method. Before preincubation, 100  $\mu\text{L}$  of the vehicle, test substance or the positive-control substance solution was added into 500  $\mu\text{L}$  of sodium phosphate buffer (pH 7.4) in the -S9 assay or 500  $\mu\text{L}$  of S9 mix in the +S9 assay, and then, 100  $\mu\text{L}$  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 sub-line passed 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<sub>2</sub> incubator (5% CO<sub>2</sub>) at 37°C. Five milliliters of the cell suspension containing  $8 \times 10^3$  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<sub>2</sub> 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-hour 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 ( $\pm$ ). 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,  $23^{\circ}\text{C} \pm 3^{\circ}\text{C}$ ; relative humidity  $55\% \pm 20\%$ ; 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 administered (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 1700g 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 1700g 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),

$\gamma$ -glutamyltranspeptidase ( $\gamma$ -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, ketone 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 420g 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  $\mu$ m; 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 Dunnett'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<sup>a</sup>

	Test substance concentration (%)	Mean number of revertants (number of colonies/plate)				
		Base-pair substitution type			Frameshift type	
		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 <sub>3</sub>	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

<sup>a</sup> 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,  $0.1\% \pm 1\%$ ; the NHE-water group,  $0.2\% \pm 1\%$ ), 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 <sup>a</sup>	-	6	100	200	3	0	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)	0 (0.0)	-
	50	-	6	94.5	200	1	0	0	0	0	0	0 (0.0)	200	0 (0.0)	0 (0.0)	-
	100	-	6	93.4	200	3	0	0	0	1	0	1 (0.5)	200	3 (1.5)	3 (1.5)	-
MMC (µg/mL)	0.1 <sup>b</sup>	-	6	78.6	200	12	20	54	0	0	0	67 (33.5)	200	0 (0.0)	0 (0.0)	+

<sup>a</sup> Negative control (sterile water for injection).<sup>b</sup> 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 <sup>a</sup>	+	6	100	200	2	0	1	0	0	0	1 (0.5)	200	0 (0.0)	0 (0.0)	-
	25	+	6	109.4	200	1	0	0	0	0	0	2 (1.0)	200	1 (0.5)	1 (0.5)	-
	50	+	6	88.8	200	3	0	1	0	0	0	1 (0.5)	200	2 (1.0)	2 (1.0)	-
	100	+	6	89.3	200	0	0	1	0	0	0	1 (0.5)	200	1 (0.5)	1 (0.5)	-
CP (µg/mL)	12.5 <sup>b</sup>	+	6	97.0	200	5	7	25	0	0	0	30 (15.0)	200	0 (0.0)	0 (0.0)	+

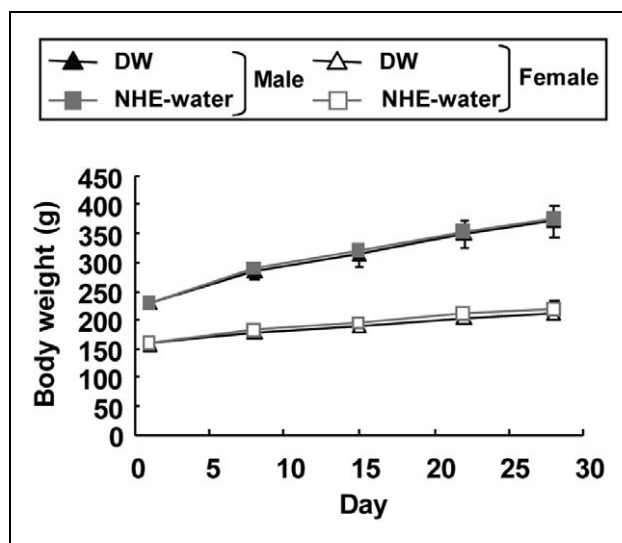
<sup>a</sup> Negative control (sterile water for injection).<sup>b</sup> Positive control (CP; cyclophosphamide). See materials and methods for abbreviations.

**Table 4.** Chromosome analysis of Chinese hamster cells

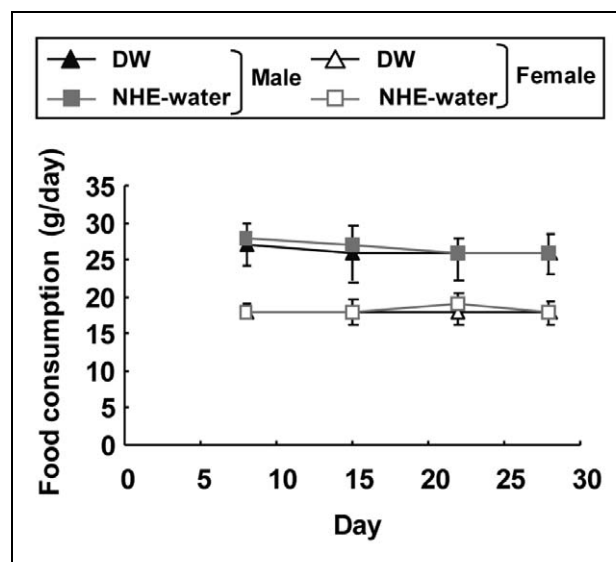
Group	Concentration (%)	S9 mix	Time of exposure (hours)	Relative cell growth (%)	Number of Cells analyzed	Number of cells with structural aberrations							Number of cells with aberrations except gap (%)	Number of cells analyzed for polyploid	Number of polyploidy cells (%)	Final judgement
						gap	ctb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

<sup>a</sup> Negative control (sterile water for injection).

<sup>b</sup> 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  $\pm$  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  $\pm$  SD.

**Table 5.** Hematological evaluation in rats treated orally with DW or NHE-water for 28 days<sup>a</sup>

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

<sup>a</sup> Values shown as mean  $\pm$  SD. See materials and methods for abbreviations

<sup>b</sup> 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  $\pm$  6 U/L; the NHE-water group, 69  $\pm$  5 U/L, ALT; the control group, 29  $\pm$  2 U/L; the NHE-water group, 28  $\pm$  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<sup>a</sup>

	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

<sup>a</sup> 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<sup>a</sup>

	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 <sup>b</sup>	71 ± 11
ALT (U/L)	29 ± 2	24 ± 5	28 ± 2 <sup>b</sup>	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

<sup>a</sup> Values shown as mean ± SD. See Materials and Methods for abbreviations.

<sup>b</sup> 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<sup>a</sup>

	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

<sup>a</sup> Values shown as mean ± SD. See materials and methods for abbreviations.

**Table 9.** Summary of organ weight/body weight ratio (% of body weight)<sup>a</sup>

	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

<sup>a</sup> 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  $\times$  60 kg = 1200 mL/day.

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