Original Article

DOI: 10.5582/ddt.2023.01080

Single intratracheal administration toxicity study on safety of vapor inhalation of electrolyzed reduced water in rats

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SUMMARY The effects of acute intratracheal administration of electrolyzed reduced water (ERW; alkaline electrolyzed water) were investigated in rats. In this study, no deaths or near-deaths were recorded in either group, namely those treated with ERW or purified water (maximum doses of 900 mg/kg). The main symptoms observed in the rats were decreased spontaneous movements and abnormal breath sounds, which were considered to be transient symptoms caused by intratracheal administration. In addition, low values of alkaline phosphatase, total protein and lactate dehydrogenase were found in BALF tests, but these values were considered to be of low toxicological significance, since they are usually high in the presence of lung inflammation or cellular damage. This suggests that the alkalinity of ERW partially contributes to broken peptide bonds in proteins. There were no significant increases in bronchoalveolar lavage fluid protein in either group. ERW did not cause an increase in the influx of neutrophils, eosinophils, basophils, or lymphocytes, suggesting that intratracheal administration of ERW did not cause lung inflammation. ERW did not cause abnormalities in the body or pathological changes in the lungs. Aggregates of alveolar macrophages, as a measure of inflammation, were observed in both groups. These may be transient symptoms due to intratracheal administration, not due to ERW toxicity. This study confirmed the safety of intratracheal ERW infusion and demonstrated the low risk of acute toxicity for inhalation exposure to ERW aerosol or vapor. Therefore, ERW may be an effective air purifier against viruses or bacteria.

Keywords alkaline electrolyzed water, acute toxicity, intratracheal instillation

1. Introduction

Electrolyzed reduced water (ERW) is a functional water that has a high pH, negative oxidative redox potential, and a high concentration of dissolved hydrogen and is produced near the cathode during the electrolysis of water. It possesses reactive oxygen species-free radical scavenging activity, conferred by the dissolved H_2 (*1*).

Several studies have shown that ERW has antibacterial activity against some bacteria (*2,3*) and antiviral effects against some types of viruses (*4*). It was recently reported that ERW has antiviral activity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the etiological agent responsible for coronavirus infectious disease, emerged in 2019 (COVID-19) (*5,6*). Potent antibacterial and antiviral activities are the motivation for further development of ERW as a monotherapy or in combination with other effective agents against infection.

There is a growing consensus that improved disinfection of environmental surfaces is needed for effective infection prevention, and traditional manual disinfection techniques are often suboptimal because of various personnel issues (*7*). Therefore, automated decontamination technologies, including aerosol (*8*) and vaporized disinfectant (*9*), are gaining more attention for the reduction of bacterial surface contamination. Similarly, ERW aerosols or vapors may be increasingly applied for air purification and to prevent the spread of viral infections, including SARS-CoV-2. Although the safety of ERW for drinking has been confirmed (*10,11*), the safety of inhaling ERW as an aerosol or vapor has not been reported. In this study, we examined the toxicity of a single intratracheal dose of ERW in rats.

2. Materials and Methods

2.1. Reagents

The ERW (product name, S-100® Medical grade) was supplied by A.I. System Products Corp. (Japan). Purified water was purchased from Takasugi Pharmaceutical (Japan).

2.2 Animals

Sprague Dawley rats were obtained from Charles River Japan (Production Plant: Hino Breeding Center, Japan). In this study, twelve 7-week-old male rats weighing 256.8-269.4 g and free of viral pathogens and parasites were used. The rats were housed in ventilated stainless cages, one per cage, with 12-hr light-dark cycles. They were provided air ventilated 10-15 times/hour, irradiated solid feed MF (Oriental Yeast Co., Ltd, lot number 200819), and tap water with sodium hypochlorite (Purax, OYALOX Co. Ltd., Japan).

All animal procedures were carried out in accordance with the "Animal Experiment at Chemicals Evaluation Research Institute (CERI) Hita" prepared by this testing facility with reference to "Act on Welfare and Management and of Animals" (Act No. 105,), 1973 "Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals" (Ministry of the Environment, 2006), "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Research Institutions under the jurisdiction of the Ministry of Health, Labour and Welfare" (Ministry of Health, Labor and Welfare, 2006), "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Agriculture, Forestry, and Fisheries" (Ministry of Agriculture, Forestry and Fisheries, 2006), "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions" (Ministry of Education, Culture, Sports, Science and Technology, 2006), and "Guidelines for Proper Conduct of Animal Experiments" (Japan Academic Conference, 2006).

2.3. Time-course study design

On day 0, rats were anesthetized with isoflurane and intratracheally instilled with 0.9 mL/kg of ERW or vehicle (purified water) using a 1 mL syringe (Terumo® Syringe, Terumo, Japan) fitted with MicroSprayer® (IA-1B R, PennCentury™, USA) (Table 1). All animals were observed continuously for 10 min immediately after dosing, then again at 30 min after dosing, and then hourly for 1 to 6 h after dosing. For the first 14 days after dosing, the animals were observed once a day in the morning. Body weight was measured before dosing on day 0 and then on days 1, 3, 7, and 14 using an electronic top-loading balance (Sartorius LP2200S QUINTIX5101-1S, Sartorius AG, Germany).

All animals were humanely euthanized on day 14 under anesthesia with isoflurane followed by exsanguination. The left lung was clamped while bronchoalveolar lavage was performed on the right lung lobes, and bronchoalveolar lavage fluid (BALF) was retained for analysis. The first fraction of the bronchoalveolar lavage was obtained by inflating the right lung with 4 mL of phosphate-buffered saline (PBS), withdrawing, and repeating the process a second time with the same 4 mL of PBS. Histopathological assessment of lung inflammation and injury were performed.

2.4. Analysis of the BALF cells

The bronchoalveolar lavage cells were counted using an XT-2000i (Sysmex, Japan) multi-item automated blood cell analyzer. The BALF was centrifuged at 1,500 rpm for 10 min. The cell pellet was washed and resuspended in 1 mL of PBS on a slide glass, and then 25 or 50 μL of the resuspended cell solution was centrifuged at 1,000 rpm for 2 min. Leukocyte counts (macrophages, neutrophils, eosinophils, basophils, and lymphocytes) were examined on smears stained using the May-Grünwald-Giemsa method. From each sample, 200 cells were counted.

The supernatant obtained after centrifugation was used to examine the alkaline phosphatase (ALP), total protein, albumin, and lactate dehydrogenase (LDH) using a Hitachi Automatic Analyzer 3500 (Hitachi High-Tech, Japan). ALP activity was determined using the p-nitrophenyl phosphate method standardized by the Japan Society of Clinical Chemistry. LDH activity was quantified using the UV method standardized by the Japan Society of Clinical Chemistry. The concentration of the total protein was measured using the pyrogallol Red method, and albumin was quantified by immunonephelometry.

2.5. Pathological examination

Table 1. The composition of animal groups

group	Administration dose (mg/kg)	ERW solution concentration (w/v%)	Administration volume (mL/kg)	Number of Animals
Control group	0	0	0.9	6
Test substance group	900	100	0.9	6

After the euthanasia of rats, gross observations were made on the body surface, openings, subcutaneous, thoracic, abdominal, and pelvic cavities and their contents. The trachea, lungs, and posterior mediastinal lymph nodes were then removed from the rats. The left lung was weighed and fixed by infusion of 10% neutralized buffered formalin. Multiple thin transverse slices were made through the left lung. These slices were embedded in paraffin and stained with hematoxylin and eosin (H&E). Selected lung sections were observed using a light microscope, and their general morphologies were noted.

2.6. Statistical analyses

The data were analyzed using Microsoft EXCEL 2016. The results were expressed as the mean ± standard deviation. Groups were compared using an unpaired two-tailed Student's *t*-test or Welch's *t*-test according to the results of the *F*-test, and a *p*-value of < 0.01 or 0.05 was considered significant, respectively.

3. Results

3.1. General clinical observations

In this study, there were no deaths or near-deaths in either group. The main symptoms were decreased spontaneous movements at a rate of 100% (6/6) and 100% (6/6) and abnormal breath sounds at a rate of 50.0% (3/6) and 33.3% (2/6) in rats treated with ERW and purified water, respectively (Table 2). The abnormal breath sounds disappeared within 1 h after dosing, and the decreased spontaneous movements disappeared 1 day after dosing. From 1 to 14 days after dosing, no abnormalities were observed in either group.

Abnormal respiratory sounds and decreased spontaneous locomotion were recorded not only in the group exposed to ERW but also in the negative control group and disappeared within 1 day. Therefore, these symptoms were considered to be transient responses caused by intratracheal administration, not due to ERW toxicity.

3.2. Body weight

The average body weight in the groups treated with ERW and purified water increased by 14.2% (from 259.3 ± 3.0 to 367.7 ± 22.5) and 14.0% (from 264.2 ± 4.6 to 368.9 ± 26.1) 14 days after administration, respectively (Figure 1).

3.3. BALF cells differentials

Cell differentials were performed on BALF cells to further assess inflammation (Table 3). ERW did not cause an increase in the influx of neutrophils, eosinophils, basophils, or lymphocytes. This is consistent with the observation of BALF proteins, further indicating that intratracheal administration of ERW did not cause lung inflammation.

3.4. BALF chemical examinations

Protein mediators were assessed in BALF to evaluate the inflammation and immune response (Figure 2). There were no significant increases in BALF protein in groups exposed to 900 mg/kg of ERW, compared with the control. These observations suggest that intratracheal injection of ERW did not cause inflammation in the lungs. However, administration of ERW did cause a significant decrease in total proteins, ALP, and LDH compared with the control. This suggests that the alkalinity of ERW was partially responsible for breaking peptide bonds in proteins.

3.5. Pathological examination

Lung (left) weight was measured after the euthanasia of rats (Table 4). The average lung weights in the groups treated with ERW and purified water were 0.523 ± 0.023 and 0.530 ± 0.045, respectively. There was no significant difference in lung weights between the two groups.

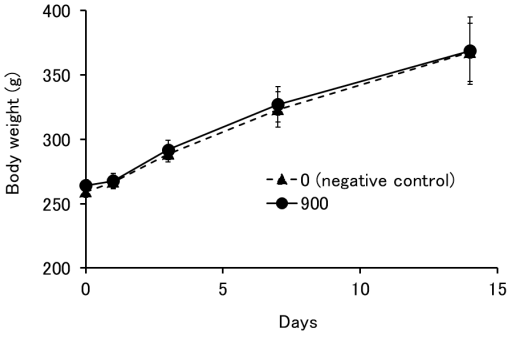

Figure 1. Single intratracheal administration toxicity study in rats. Summary of body weights. Values are shown as the mean ± S.D. *Significantly different from vehicle control at $p < 0.05$.

Table 2. Single intratracheal administration toxicity study in rats. Summary of general clinical observations

Signs	Number of cases		
	Dose (mg/kg)	0 (negative control) ($n = 6$)	900 ($n = 6$)
Deaths and near-deaths		0	0
Abnormal respiratory sound		2	3
Decreased spontaneous locomotion		6	6

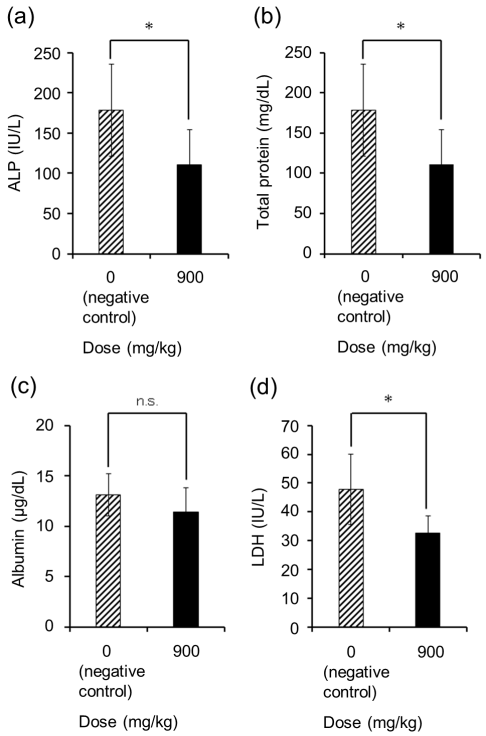

Figure 2. Single intratracheal administration toxicity study in rats. Summary of BALF chemical examinations. a): ALP (IU/L), b): Total protein (mg/dL), c): Albumin (μg/dL), d): LDH (IU/L). Values are shown as the mean ± S.D. *Significantly different from vehicle control at $p < 0.05$.

3.6. Gross observations and H&E-stained lung tissue sections

Gross observations and H&E-stained lung tissue sections were evaluated (Table 5). ERW at 900 (mg/kg) dose did not cause abnormalities on the body surface, openings, subcutaneous, thoracic, abdominal, and pelvic cavities, and pathological changes in the lung. Aggregates of alveolar macrophages, as a measure of inflammation, were observed in ERW and purified water-exposed groups. Very slight aggregates of macrophages were observed at a rate of 16.7% (1/6) and 50% (3/6) respectively, and slight aggregates were observed at a rate of 33.3% (2/6) and 16.7% (1/6) respectively in ERW-exposed and control groups. These aggregates of macrophages were inferred to be transient symptoms due to intratracheal administration, not due to ERW toxicity.

4. Discussion

In this study, the upper limit of the dose for intratracheal administration was set at 900 mg/kg, based on the draft guidelines on risk assessment methods for the indoor use of insecticides as OTC drugs and quasi-drugs issued by the Ministry of Health, Labour and Welfare in Japan (*12*). Furthermore, the reasonable dose for the upper limit of the

Table 3. Single intratracheal administration toxicity study in rats. Summary of BALF cell examinations

		BALF cell examinations		
		Dose (mg/kg)	0 [a] (negative control) ($n = 6$)	900 ($n = 6$)
Total cells	($\times 10^2$/μL)		1.27 ± 0.55	1.23 ± 0.45
Differentiation of leukocyte	Macrophages (%)		99.58 ± 0.80	99.08 ± 2.01
	Neutrophils (%)		0.08 ± 0.20	0.00 ± 0.00
	Eosinophils (%)		0.00 ± 0.00	0.08 ± 0.20
	Basophils (%)		0.00 ± 0.00	0.00 ± 0.00
	Lymphocytes (%)		0.33 ± 0.61	0.83 ± 1.81

Values are shown as the mean ± S.D. [a]In the control group, 0.9 mL/kg of purified water was administered.

Table 4. Single intratracheal administration toxicity study in rats. Summary of organ weights

	weights		
	Dose (mg/kg)	0[a] (negative control) ($n = 6$)	900 ($n = 6$)
Lung (left) (g)		0.523 ± 0.023	0.530 ± 0.045
(g/100g)		(0.140 ± 0.011)	(0.143 ± 0.012)
Final body weight (g)		367.7 ± 23.0	368.9 ± 26.1

Values are shown as the mean ± S.D. [a]In the control group, 0.9 mL/kg of purified water was administered.

Table 5. Single intratracheal administration toxicity study in rats. Macroscopic examination and histopathological examinations

			Number of cases	
		Dose (mg/kg)	0 [a] (control) ($n = 6$)	900 ($n = 6$)
Gross observations	Abnormalities		0	0
H&E-stained lung tissue sections	abnormalities		0	0
	aggregates of alveolar	very slight	3	1
	macrophages	slight	1	2

Values are shown as the mean ± S.D. [a]In the control group, 0.9 mL/kg of purified water was administered.

dose for intratracheal administration was determined as follows. The respiratory volume in rats has been reported to be 0.27 m^3/day in male Sprague Dawley rats weighing 267 g (*13*). The respiratory volume is estimated to be 0.01125 m^3/hr. Based on the limiting concentration in the acute inhalation toxicity test of 5 mg/L (5,000 mg/m^3) and the respiratory volume of the rats, and assuming that all the inhaled substance reaches the lungs, the amount is estimated to be equivalent to 5,000 mg/m^3 × 0.01125 m^3/h = 56.25 mg/h. In addition, if the exposure time of the acute inhalation toxicity test is 4 h, the maximum amount of exposure to the lungs of rats is estimated to be 56.25 mg/h × 4 h = 225 mg. In other words, if the body weight of a rat is 267 g, assuming that all particles in the air reach the lungs by respiration, the maximum dose is calculated to be approximately 843 mg/kg. Therefore, the maximum dose of 900 mg/kg is considered to be a reasonable dose above the limiting concentration of 5 mg/L in the acute inhalation toxicity study.

The ERW (product name, S-100® Medical grade) used in this study is manufactured by the high-voltage electrolysis of water containing natural salt. It contains mineral ions, namely Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^- (*14*). It has a high pH (12.1), low surface tension (62.3 dyne/cm), and BAP (4,905 μmol/L) (*5*).

No deaths or near-deaths occurred in the animals during the dose-finding study or in the first step when ERW was administered at 900 mg/kg. Furthermore, according to the Globally Harmonized System of Classification and Labelling of Chemicals, the LC50 cut-off value for acute toxicity of ERW in rats under the conditions of this study, when converted from intratracheal administration to inhalation exposure, corresponds to ∞, and is considered to be "Not applicable to classification". This also suggests that there are no toxic effects for the intratracheal administration of ERW. Therefore, considering the potent anti-virus and anti-microbial activities, further development of ERW as an effective air purifier by spraying against virus or microbial infection is warranted.

Funding: A portion of this study was supported by Iwatani Corporation Japan.

Conflict of Interest: The electrolyzed reduced water (ERW, product name S-100® Medical grade) used in this study was manufactured by A. I. System products, Corp. Yoshinao Okajima and Masahiro Okajima are employees of A. I. System products, Corp.

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Received October 7, 2023; Revised December 5, 2023; Accepted December 15, 2023.

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Released online in J-STAGE as advance publication December 22, 2023.