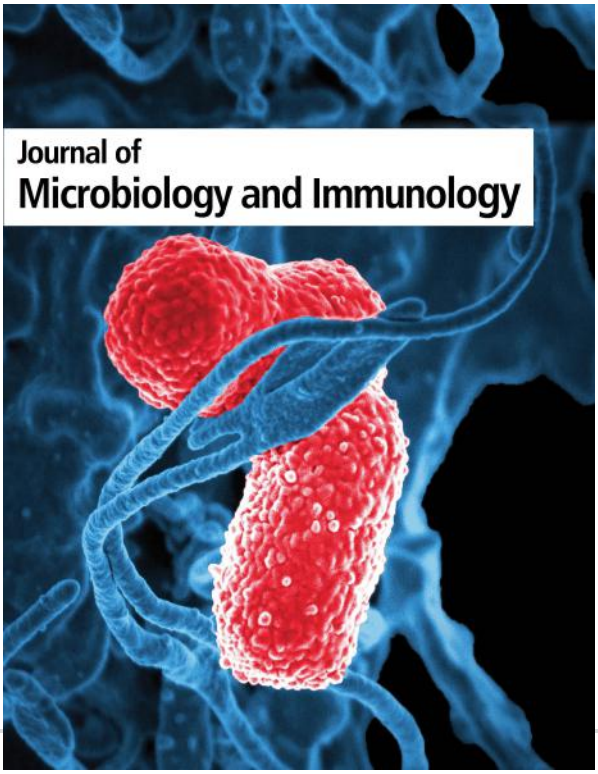

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Reduction of severe acute respiratory syndrome Coronavirus - 2 by admissible concentration of Ozone Gas and Water

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is causing the global coronavirus disease 2019 (COVID-19) pandemic. Because complete elimination of SARS-CoV-2 appears difficult, decreasing the risk of transmission is important. Treatment with 0.1 and 0.05 ppm ozone gas for 10 and 20 hr, respectively, decreased SARS-CoV-2 infectivity by about 95%. The magnitude of the effect was dependent on humidity. Treatment with 1 and 2 mg/L ozone water for 10 s reduced SARS-CoV-2 infectivity by about 2 and 3 logs, respectively. Our results suggest that low-dose ozone, in the form of gas and water, is effective against SARS-CoV-2.

1 INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped virus that belongs to the Coronaviridae family and has a positive-strand RNA as its genome. It emerged in late 2019 in Wuhan, China, and rapidly spread globally. Because SARS-CoV-2 has high sequence similarity with some bat coronaviruses, it is assumed that bats are the natural reservoir, and that it was transmitted to humans via an intermediate host, such as the pangolin. SARS-CoV-2 infection causes flu-like symptoms, including fever, cough, pharyngeal pain, and a sense of fatigue during the early stages. Some patients also go on to develop pneumonia, which can progress to acute respiratory distress

syndrome and other serious complications. The disease caused by SARS-CoV-2 is known as coronavirus disease 2019 (COVID-19).[1]

The world has not experienced such a pandemic for approximately 100 years, since the Spanish flu outbreak in 1918. As of 14 September 2020, 28.6 million people have been infected with SARS-CoV-2 and 917,000 have died.[2] The outcome of SARS-CoV-2 infection varies by age, underlying health conditions, the availability of medical care, and nationality, and the mortality rate is estimated at 2–3%. The basic reproduction rate (R0) in Wuhan was reported at 2–3,[3] but can be decreased markedly by appropriate countermeasures, such as air ventilation, hand washing, and ethanol spraying.

Ozone (O_3) is composed of three oxygen atoms. It is present at 2–10 ppm in the ozone layer of the stratosphere, and at <0.05 ppm near the ground. Ozone is unstable and highly reactive, having a half-life of about 1 hr under ambient conditions; also, it oxidizes certain substances. Ozone is a strong oxidant that produces hydroxyl radicals in the presence of water. Hydroxyl radicals have the highest oxidative power among all reactive oxygen species, so the oxidative effect of ozone gas is highest under humid conditions. Ozone gas and water are frequently used to disinfect rooms (floors, walls, ceilings, furniture, and air), instruments, hands, clean and sewage water, and food (vegetables, fruits, fish, and meat). Ozone inactivates a variety of microorganisms, such as bacteria, fungi, parasites, and viruses.[4, 5] Ozone damages viral structural molecules, such as envelope lipids, glycoproteins, and capsid proteins, thereby inactivating the virus.

Because the effect of ozone on SARS-CoV-2 is unknown, we evaluated the effect of low-dose ozone gas on SARS-CoV-2. The infectious SARS-CoV-2 titer decreased over time in the presence of 0.1 or 0.05 ppm ozone gas. Also, low-concentration ozone water reduced SARS-CoV-2 infectivity. Therefore, ozone at a nontoxic concentration can be used to suppress SARS-CoV-2 transmission.

2 MATERIALS AND METHODS

2.1 Cell culture and reagents

VeroE6/TMPRSS2 cells express the transmembrane serine protease 2 gene and were obtained from the Japanese Collection of Research Bioresources Cell Bank. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai-Tesque), supplemented with 5% fetal bovine serum (FBS) and penicillin–streptomycin (Sigma-Aldrich). SARS-CoV-2 (SARS-CoV-2/Hu/DP/Kng/19-020, isolated from a throat swab of a patient on the cruise ship *Diamond Princess*) was obtained from the Kanagawa Prefectural Institute of Public Health, Japan. The virus was amplified by infecting VeroE6/TMPRSS2 cells in a 175 cm^2 culture bottle in DMEM supplemented with 1% FBS. When 100% of the cells showed a cytopathic effect, the culture medium was harvested, centrifuged at low speed, and filtered. Amplified virus was titrated using VeroE6/TMPRSS2 cells.

2.2 Ozone gas/water administration

For ozone gas administration at a constant predefined concentration, we used a sophisticated regulation system (Figure 1, supported by Tamura TECO Co. Ltd). For ozone gas treatment, an ozone gas generator, ozone gas monitor, and thermo-hygrometer probe were placed in a transparent acrylic container (Figure 1a). As a control, we used a similar container without the ozone gas generator. According to the ozone gas concentration, the power to the gas generator was switched on and off by

the control system to maintain a constant ozone concentration (Figure 1b). A water reservoir was used to humidify the air in the container (Figure 1c).

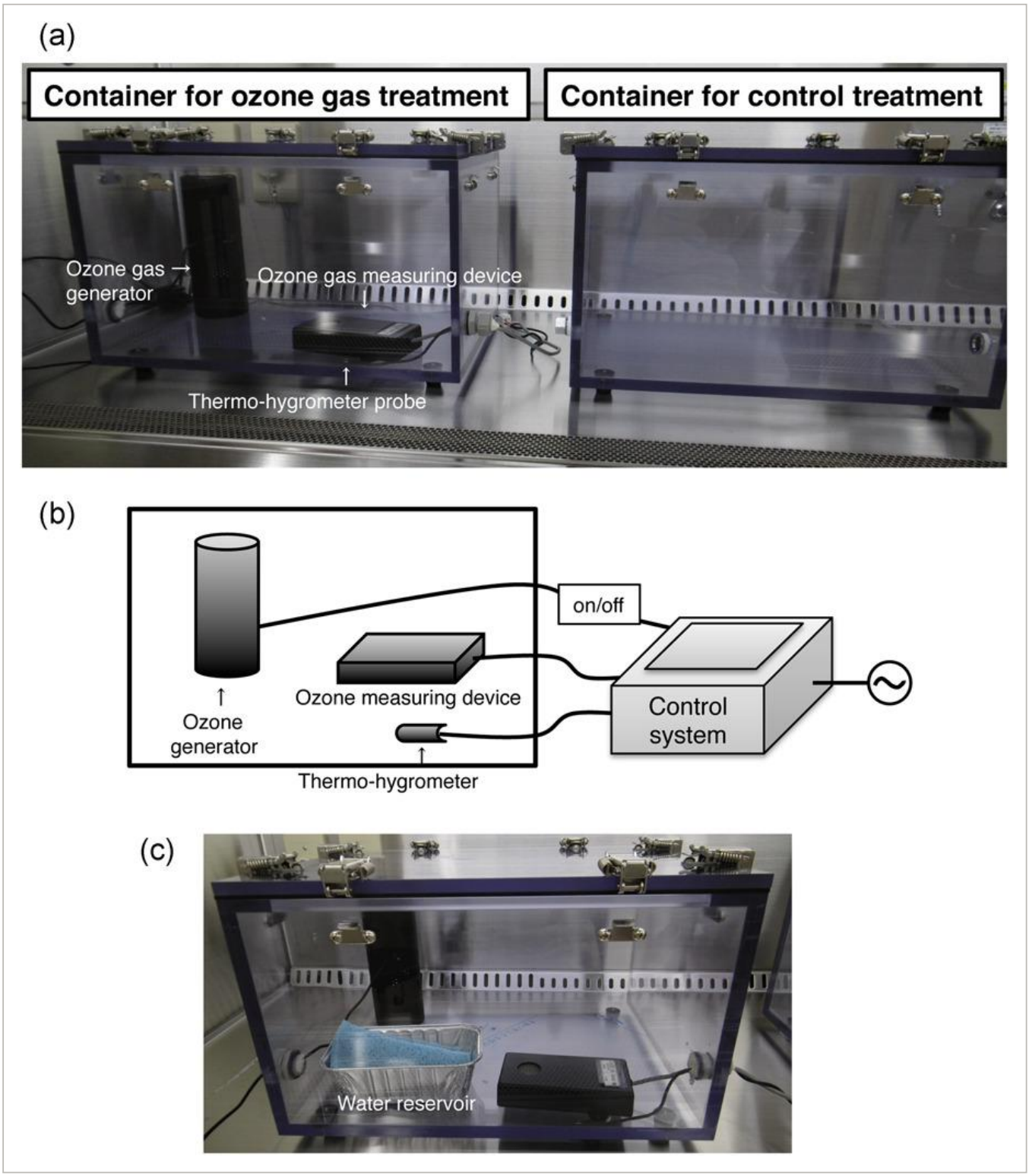

Figure 1

Equipment for ozone gas administration. (a) Two containers were prepared: one for ozone gas treatment and another for control treatment. (b) Schematic diagram of the ozone gas equipment. The control system is switched on or off according to the data obtained by the gas-measuring device. (c) Water reservoir for humidity [Color figure can be viewed at wileyonlinelibrary.com]

Stainless-steel carriers were formed into disks 2 cm in diameter. Virus solution (50 µL) was dropped and spread onto the carriers and air-dried. The carriers were placed in the containers, and ozone gas was administered under the indicated conditions. Virus was collected by suspending the carriers in DMEM containing 2% FBS (500 µL).

Ozone water was produced using the ICLEAN Minnie (Tersano Co. Ltd), and the concentration was assayed by measuring light absorbance using an ozone colorimeter (C105; Eutech Instruments Co.). Next, the virus solution (1 µL) was mixed with ozone water (100 µL). After 10 s, 899 µL of DMEM supplemented with 20% FBS was added and mixed thoroughly.

Medium containing virus was serially diluted 10-fold with fresh DMEM containing 2% FBS, and dispensed onto VeroE6/TMPRSS2 cells in a 96-well culture plate. The tissue culture infectious dose 50 ($TCID_{50}$) was calculated on the appearance of cytopathic effects. Two or three independent experiments were carried out for each condition.

For ozone gas experiments, the concentration-time (CT) value was defined as the ozone gas concentration (ppm) multiplied by the duration of administration (min). The CT 60 samples were collected after incubation in ozone treatment container 10 and 20 hr, for the 0.1 and 0.05 ppm ozone gas conditions, respectively. The CT 0 samples were incubated in the control container for 10 and 20 hr for the 0.1 and 0.05 ppm ozone gas conditions, respectively. For the CT 24 samples, SARS-CoV-2 was incubated in the ozone gas container for 4 and 8 hr, for 0.1 and 0.05 ppm, respectively, and then transferred to the control container for the remainder of the experiment. Likewise, CT 42 samples were incubated with ozone gas for 7 and 14 hr, for 0.1 and 0.05 ppm, respectively, and then settled in the control container for the rest of the time.

3 RESULTS

3.1 Ozone gas treatment

First, we administered ozone gas at 0.1 ppm, which is the permissible concentration for humans defined by the Japan Society for Occupational Health (Table 1). A water reservoir was placed in both containers to humidify the air. Ozone gas treatment was started when the humidity reached 75%. The average humidity was about 80%. When the CT value reached 24 (4 hr), SARS-CoV-2 infectivity decreased by 73.2% compared with the control. The virus was inactivated by 86.7% and 95.4% at CT 42 and 60, respectively. Next, we evaluated 0.05 ppm ozone gas, the maximum tolerated concentration according to the United States Food and Drug Administration, under humid conditions (Table 2). We found 60.9%, 82.4%, and 94.3% reductions in the SARS-CoV-2 titer at CT 24, 42, and 60, respectively.

Table 1. Data obtained under the 0.1 ppm ozone gas humid conditions

CT	Duration[a]	$TCID_{50}$/mL	Average	% reduction	$LogTCID_{50}$/mL	Average	Log reduction	*P*
0	0	3.16×10^6	3.67×10^6	0.0	6.50	6.56	0.00	
		4.68×10^6			6.67			
		3.16×10^6			6.50			

CT	Duration[a]	$TCID_{50}$/mL	Average	% reduction	$LogTCID_{50}$/mL	Average	Log reduction	*P*
24	4	1.48×10^6	9.82×10^5	73.2	6.17	5.95	0.61	0.0179
		1.00×10^6			6.00			
		4.68×10^5			5.67			
42	7	3.16×10^5	4.87×10^5	86.7	5.50	5.67	0.89	0.0013
		6.76×10^5			5.83			
		4.68×10^5			5.67			
60	10	1.48×10^5	1.70×10^5	95.4	5.17	5.22	1.33	0.0001
		2.14×10^5			5.33			
		1.48×10^5			5.17			

[a] Incubation time in ozone container (hours).

CT, concentraion-time; $TCID_{50}$, tissue culture infectious dose 50.

Table 2. Data obtained under the ozone gas 0.05 ppm humid conditions

CT	Duration[a]	$TCID_{50}$/mL	Average	Percent reduction	$LogTCID_{50}$/mL	Average	Log reduction	*P*
0	0	1.48×10^6	1.70×10^6	0.0	6.17	6.22	0.00	
		1.48×10^6			6.17			
		2.14×10^6			6.33			
24	8	3.16×10^5	6.64×10^5	60.9	5.50	5.78	0.45	0.046
		1.00×10^6			6.00			
		6.76×10^5			5.83			
42	14	4.68×10^5	2.98×10^5	82.4	5.67	5.44	0.78	0.003
		2.14×10^5			5.33			
		2.14×10^5			5.33			
60	20	1.45×10^5	9.71×10^4	94.3	5.16	4.94	1.28	0.001
		4.68×10^4			4.67			
		1.00×10^5			5.00			

[a] Incubation time in ozone container (hours).

CT, concentraion-time; $TCID_{50}$, tissue culture infectious dose 50.

At ambient humidity (55%), 0.1 ppm ozone gas nonsignificantly decreased infectivity, by 68.4% at CT 60 (Table 3). Therefore, humidity is important for reducing SARS-CoV-2 infectivity using ozone gas.

Table 3. Data obtained under the ozone gas 0.1 ppm ambient conditions

CT	Duration[a]	$TCID_{50}$/mL	Average	Percent reduction	$LogTCID_{50}$/mL	Average	Log reduction	*P*
0	0	2.14×10^6	3.41×10^6	0.0	6.33	6.50	0.00	
		4.68×10^6			6.67			
24	4	2.14×10^6	1.81×10^6	46.9	6.33	6.25	0.25	0.315
		1.48×10^6			6.17			
42	7	1.00×10^6	1.57×10^6	54.0	6.00	6.17	0.34	0.293
		2.14×10^6			6.33			
60	10	6.76×10^5	1.08×10^6	68.4	5.83	6.00	0.50	0.173
		1.48×10^6			6.17			

[a] Incubation time in ozone container (hours).

CT, concentraion-time; $TCID_{50}$, tissue culture infectious dose 50.

Inactivation of SARS-CoV2 by ozone gas was correlated with the CT value, but not just with the ozone concentration (Figure 2). There was no significant difference in the reduction rates in viral titer between 0.1 and 0.05 ppm ozone under humid conditions (black diamond and black rectangle). Therefore, a 50% reduction in ozone concentration can be compensated for by doubling the treatment duration. Also, humidity markedly affected the effectiveness of ozone gas (Figure 2).

Figure 2

Relationship of the CT value of ozone gas with the tissue culture infectious dose 50 ($TCID_{50}$) value. The average log $TCID_{50}$/mL values of two or three independent experiments are shown

3.2 Ozone water treatment

We next tested the effect of ozone water on SARS-CoV-2 infectivity. Addition of 1.0 and 2.0 mg/L ozone to water for 10 s reduced infectivity by about 2 and 3 logs, respectively (Table 4). Treatment with 0.4 and 0.7 mg/L ozone water resulted in a 94.2% and 99.5% decrease, respectively (Table 5). Although the slope of the functions in Experiments 1 and 2 were slightly different (Figure 3), efficacy of ozone water was seen in both.

Table 4. Ozone water data for Experiment 1

Conc (mg/L)	$TCID_{50}$/mL	Average	Percent reduction	$LogTCID_{50}$/mL	Average	Log reduction	*P*
0.0	1.78×10^6	1.63×10^6	0.0	6.25	6.21	0.00	
	1.33×10^6			6.13			
	1.78×10^6			6.25			
1.0	7.50×10^3	1.14×10^4	99.3	3.88	4.04	2.17	<0.001
	1.33×10^4			4.13			
	1.33×10^4			4.13			
2.0	5.62×10^2[a]	8.80×10^2[a]	99.9	2.75[a]	2.92[a]	3.29	<0.001
	7.50×10^2[a]			2.88[a]			
	1.33×10^3[a]			3.12[a]			

[a] Because only a few wells exhibited CPE, $TCID_{50}$ could not be determined precisely. The data shown here are estimated maximum values.

$TCID_{50}$, tissue culture infectious dose 50.

Table 5. Ozone water data for Experiment 2

Conc (mg/L)	$TCID_{50}$/mL	Average	Percent reduction	$LogTCID_{50}$/mL	Average	Log reduction	*P*
0.0	1.33×10^7	1.48×10^7	0.0	7.13	7.17	0.00	
	1.33×10^7			7.13			
	1.78×10^7			7.25			
0.4	1.00×10^6	8.54×10^5	94.2	6.00	5.92	1.25	<0.001
	5.75×10^5			5.75			
	1.00×10^6			6.00			
0.7	7.50×10^4	6.87×10^4	99.5	4.88	4.83	2.33	<0.001
	5.62×10^4			4.75			

Conc (mg/L)	$TCID_{50}$/mL	Average	Percent reduction	$LogTCID_{50}$/mL	Average	Log reduction	*P*
	7.50×10^4			4.88			
1.2	1.33×10^4	4.35×104	99.7	4.13	4.54	2.63	<0.001
	7.50×10^4			4.88			
	4.22×10^4			4.63			

$TCID_{50}$, tissue culture infectious dose 50.

Figure 3

Relationship of the ozone concentration in water with the tissue culture infectious dose 50 ($TCID_{50}$) value. The average log $TCID_{50}$/mL value of three independent experiments is shown

4 DISCUSSION

The SARS-CoV-2 pandemic has impacted public health and resulted in the loss of trillions of dollars from the economy. Therefore, safe antimicrobial agents that suppress SARS-CoV-2 transmission are needed; low-dose ozone gas is one such agent, together with alcohol, ultraviolet (UV) light, detergents, and sodium hypochlorite.[6] However, liquid agents (alcohol, detergents, and hypochlorite) are effective against SARS-CoV-2, but leave water and cannot be used against airborne virus. UV can kill viruses in the air and on surfaces. However, UV light cannot penetrate complex objects so is ineffective for sterilizing them. High-dose ozone gas kills airborne SARS-CoV-2 and can be used to disinfect complex objects, but is also toxic. Low-dose ozone gas requires high humidity for maximum effectiveness, but is nondestructive, nontoxic, and effective against airborne and fomite-borne SARS-CoV-2. Therefore, the SARS-CoV-2 disinfection method should be selected according to the specific situation. Because the half-life of ozone in water is short (10–30 min, depending on the temperature), ozone water is not storable like alcohol or detergents, but has the advantage of not leaving a residue. In addition, ozone water can be produced easily from clean water.

Low-dose ozone gas will likely be useful in hospitals, where large numbers of people congregate continuously. The structure of hospital rooms is complex and they contain desks, sofas, personal computers, air conditioners, medical devices, testing equipment, and bedding. In addition, the presence of SARS-CoV-2, in the air or on objects, is more likely in hospitals than in other settings. In combination with other precautions, such as frequent hand washing with detergent, wiping with alcohol, and UV irradiation, low-dose ozone gas and a humidifier will decrease the likelihood of infection. Also, low-dose ozone gas will likely be effective in other settings, including offices, schools, cars, ambulances, restaurants, bars, and museums.

Ozone water has been used for washing food (vegetable, fruits, and fish) and disinfecting water. In hospitals, it is used to clean the hands, mouth, eyes, and instruments. Washing hands and appliances

between examinations/treatments with ozone water would reduce the incidence of nosocomial SARS-CoV-2 infection.

We here examined whether SARS-CoV-2 in large droplets (1 µL), assuming those produced by sneezing, could be inactivated by hand washing or rubbing for a short period of time (10 s) with plenty of ozone water (100 µL), produced from a portable ozone water generator. Since ozone water inactivated the virus (Tables 4 and 5, Figure 3), portable ozone water generators may be suitable surrogates for alcohol sprays. Also, ozone water is less harmful to the skin and mucus membranes than alcohol and detergents, which, unlike ozone water, can cause allergic reactions and skin roughness.

We have demonstrated the effectiveness and safety of low-dose ozone gas and ozone water for SARS-CoV-2. However, the utility of low-dose ozone gas and water for SARS-CoV-2 prevention needs to be verified. Also, low-dose ozone gas and ozone water kill not only SARS-CoV-2, but also other pathogenic microorganisms, because the mode of action is not specific to SARS-CoV-2.

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DISCLOSURE

Tamura TECO Co. Ltd provided funding and equipment for the research. The sponsor had no role in execution, interpretation, or writing of the study.

Open Research

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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