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Microbial disinfection and safety applications of ozone generators: bactericidal effect

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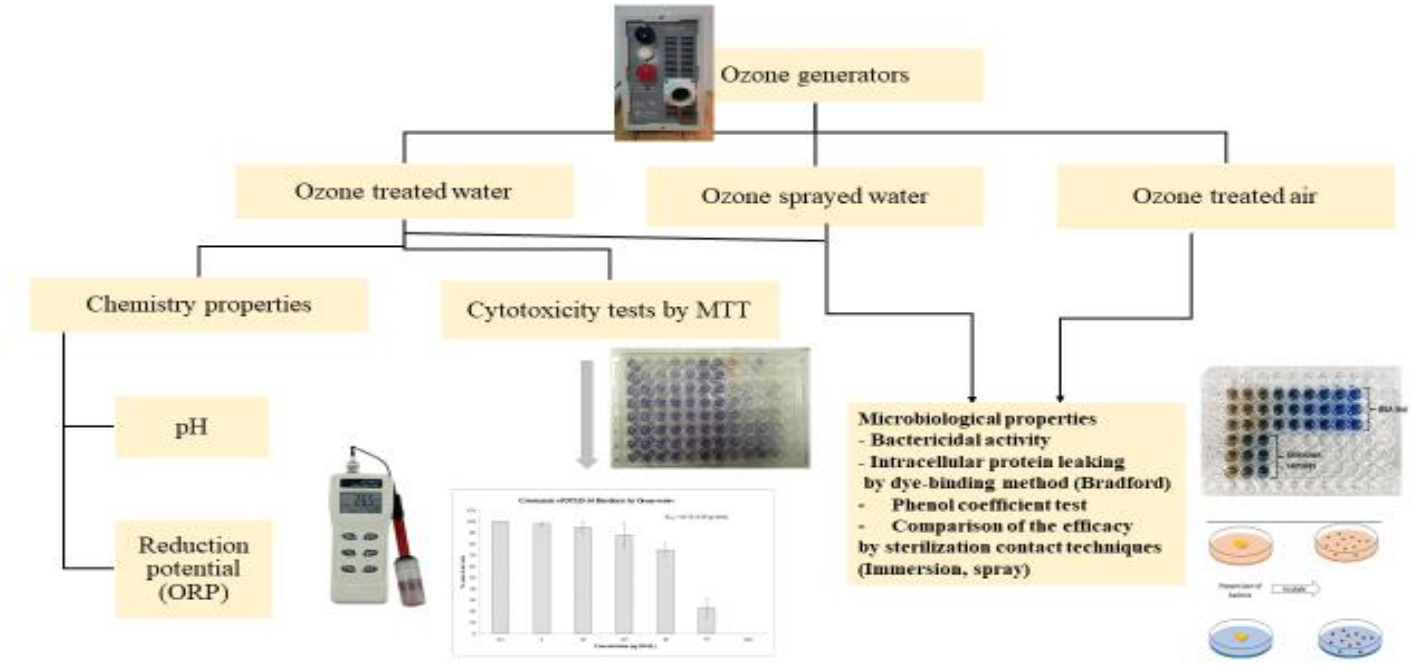
ABSTRACT

Ozone generator is one of the promising novel disinfectant technology that have recently been proposed as the alternative to conventional decontamination methods such as heat and chemical sanitizers. The objective of this research was to examine various properties of ozone generator to ensure safety and to further proper hygiene practical guideline. The tests are performed by evaluated the propertiesof chemical and microbiological on bacteria that cause clinically important pathogens at different times and cytotoxicity to Cellosaurus cell line OUMS-36 fibroblasts. Moreover, the efficacy of sterilization techniques (soaking, and spraying) of this disinfectants was compared as well. The results showed that ozone-sprayed water, ozone treated water, and ozone treated air has an ability to kill all bacteria within 1-3, 15-20 and 3-5 minutes respectively. Toxicity test and Phenol coefficient of ozone-treated water showed that it is low toxicity level and showed 0.125 times to phenol. Moreover, the use of immersion technique on smooth surface object were found to be more effective for cleaning contaminated than spraying and fabrics, which take only 15-20 minutes to disinfect. In summary, ozone generator is effective in disinfecting surfaces of objects and safe for cleaning surfaces, and other equipment. This research contributes to increasing confidence in safety and as a guideline to carry out for the better and suitable hygiene in the future.



Keywords: Disinfectant, Ozone generator, Anti-microbial, Cytotoxicity.

INTRODUCTION

The emergence of Coronavirus Disease 2019 (COVID-19), a highly contagious respiratory illness, has prompted a swift and rigorous response aimed at curbing its transmission. Preventative measures such as frequent handwashing, the implementation of barrier techniques, and the routine disinfection of surfaces have been widely recommended. However, the conventional chemical disinfectants employed for this purpose often raise concerns due to their skin irritation potential and associated safety risks.

In light of these challenges, there is a pressing need to explore alternative strategies to effectively reduce the transmission and spread of respiratory pathogens through human-to-human and human-to-environmental/surface contact. This study seeks to address this imperative by investigating innovative approaches to microbial disinfection, with a focus on their efficacy, safety, and potential to mitigate the transmission of infectious agents.

The investigation of alternative methods and/or agents for disinfection and sanitization should be prioritized, and ozone (O_3) can be a viable option with a variety of objectives. Ozone is a gas with three oxygen atoms in its natural configuration (O_3). It is an elemental form of oxygen that occurs naturally in the Earth's atmosphere and protects the planet from harmful solar ultraviolet radiation [1].O_3 can also be synthesized artificially using electricity generators. One issue with O_3 gas is its toxicity [2]. One approach to minimize this effect is to use it dissolved in water. Given O_3's toxicity and reasonable solubility in water, researchers are increasingly interested in using it as a sanitizing agent [3]. Because of O_3's solubility in water, it can react with any soluble compound or biomolecule found in biological fluids [4]. With an oxidative potential greater than most commercial disinfectants and a reaction rate faster than O_2, it has been studied in medicine and biological sciences for decades, becoming a versatile therapeutic agent that aids in the treatment of a variety of infectious diseases [5].

The purpose of this study is to determine the bactericidal ability, cytotoxicity, optimal timing, and disinfection technique for inhibiting the growth and destruction of clinically important pathogenic microorganisms with ozone sprayed water, ozone-treated water and ozone air though the testing that simulates contamination of water, plastic sheet, and fabric pad surfaces contaminated with bacteria to ensure safety and to further proper hygiene practical guideline under simulated appropriate *in vitro* laboratory conditions.

MATERIALS AND METHODS

Experimental Design

This study employed an in vitro approach, utilizing four pathogenic bacteria to evaluate the effectiveness of the ozone generator.

Microorganisms

The pathogenic bacterial strains used in this experiment were generously provided by the Faculty of Medical Technology, Rangsit University. To prepare the bacterial cultures, each strain was sub cultured on Tryptic Soy Agar (TSA) and incubated at 37°C for 24 hours to reach the logarithmic growth phase. Subsequently, isolated pure colonies were suspended in sterile 0.85% NaCl, and their absorbance was measured and adjusted to the 0.5 McFarland Standard (equivalent to 1.5 x 10^8 CFU/ml).

Chemicals and Reagents

The following chemicals and reagents were used in this study and were sourced from GIBCO BRL, Paisley, UK, and Thermo Scientific HyClone unless otherwise specified: 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), and trypsin. All other reagents utilized were of analytical grade.

Generation of Ozone

In this study, 3 types of ozone generators exposure were tested: exposure to ozone sprayed directly into the water while it was running referred to "ozone sprayed water", exposure with the water produced by the ozone machine which is referred to "ozone treated water", and exposure with the air produced by the ozone machine referred to "ozone treated air".

Chemistry property of ozone sprayed water

The pH and oxidation-reduction potential (ORP) of the ozone-treated water were determined using a Suntex TS-100 meter (Suntex Company, USA). For comparative purposes, distilled water was employed as a negative control, while sodium hypochlorite (NaClO) served as the standard disinfectant. These reference materials and methods were used to evaluate and compare the chemical properties of the ozone-treated water.

Microbiological property of ozone generator

Determination of bactericidal activity after direct exposure to ozone-sprayed water and ozone treated water

The bactericidal activity of an ozone-sprayed water and ozone-treated water were tested according to previous study [6]. For ozone sprayed water, turning on the ozone generator and spray directly into 5 mL of tested pathogenic microbial suspension water (~ 1.5 x 10^4 CFU/ml). For ozone treated water, 1 liter of distilled water was ozone sprayed directly into the water for 5 minute and mixed with 5 mL of tested pathogenic microbial suspension water (~ 1.5 x 10^4 CFU/ml) in the 1:1 ratio. Then, 100 µL of water samples were collected at difference time point (1, 3, 5, 10, 15, and 20 minutes) and spread on TSA plates for incubation at 37°C for 24 hours. After incubation period, the colony counts and quantity that grew on the culture medium was performed by manual counting method compared with the control of the cultures (0.6% sodium hypochlorite and sterile NSS as a positive control and growth control). The bacterial colony observed after an incubation period, were indicated as a colony forming units (CFUs).

The three replicates of individual experiment were performed.

Determination of bactericidal activity after direct exposure to ozone air

Ozone-treated air was tested for bactericidal activity. In brief, 100 ul of tested pathogenic microbial suspension (1.5 x 10^5 CFU/ml) was dropped onto the surface of sterile fabric sheets and allowed to dry for 1 hour. After being blown by an ozone-treated air at various time intervals (1, 3, 5, 10, 15, and 20 minutes), the contaminated fabric sheet was soaked in a 1 mL sterile 0.85 % saline solution tube and shaken for 1 minute to allow the saline solution to remove microbial from the object. The mixer was then spread onto the TSA plates and incubated for 24 hours at 37°C. Following incubation, the colony counts of bacteria growing on culture medium were manually counted and compared to control cultures (0.6 % sodium hypochlorite and sterile NSS as a positive control and growth control). A bacterial colony was displayed as a colony forming unit (CFUs). Each experiment was carried out three times.

Cell Lines and Culture Medium

Cellosaurus cell line OUMS-36 fibroblasts stock cells were maintained as monolayer cultures in DMEM supplemented with 10% inactivated FBS, 1% Antibiotic – anti-mycotic, and 1% Glutamine, in a 5% CO_2 humidified atmosphere incubator at 37°C until confluent. The stock cultures were grown in 25 cm^2 culture flasks, and the cells were dissociated using trypsin–EDTA (0.2% trypsin, 0.02% EDTA in PBS) from their culture flasks twice weekly. All experiments were carried out in 96 well micro titer plates (Nunc. Ltd., USA)

Cytotoxicity tests

For preparation of test solutions, ozone-treated water was serial concentrations such as 0.1, 1, 10, 25, 50, 75, and 100% v/v was made up with non-supplemented DMEM and sterilized by filtration. The serially dilution were prepared for carrying out cytotoxic studies.

The MTT assay was performed as described by previous study [6]. The viability of the cell was assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, each cell line (5 × 10^4 cells/well in 100 µl medium) were seeded onto 96-well micro titer plates and routinely cultured in a humidified incubator at 37°C in 5% CO_2 for 24 h. The cultivated cells were separately treated with various serially ozone water dilution (0.1 -100% v/v) andOUMS-36 fibroblasts cell line cultured in DMEM + 10% heat inactivated FBS was used as growth control. The plate was re-incubated for 24 h. Then, 10 µl of MTT dye solution (3-[4,5 -dimethylthiazol-2-yl]-2,5 -diphenyltetrazolium bromide) (5 mg/ml in PBS) was added to every well and re-incubated for 4 h. After removing un-transformed MTT reagent, 100 µl of DMSO was added to dissolve the formed formazan crystals and the plate was further incubated for 5 min at room temperature. Amount of formazan was determined by measuring the optical density at a wavelength of 570 nm using a Micro-plate reader (Biotek: Synergy HT). All experiments were carried out 3 times. The absorbance reading was taken to calculate the percentage of cell survival as follow:

$$(\%\ \text{cell viability}) = \frac{(\text{OD sample}) \times 100}{\text{OD negative control}}$$

$$(\%\ \text{Cytotoxicity}) = \frac{100 - (\text{ODsample}) \times 100}{\text{OD negative control}}$$

The data were expressed as the concentration of sample required to kill 50% (IC50) of the cells compared to the controls.

Measurement of intracellular protein leaking by dye-binding method (Bradford)

To test intracellular protein leaking from interested bacteria by ozone-sprayed water was performed by 5 mL of tested pathogenic microbial suspension (1.5 x 10^8 CFU/ml) was exposed to ozone-sprayed water at different time points (1, 3, 5, 10, 15, and 15 min). At the end of each time period, 10 µL of suspension was mixed with 200 µL of coomassie dye, and the color produced by the coomassie dye-protein interaction was measured using the dye-binding Bradford method (Bradford M., 1976). The optical density (OD) was measure at a wavelength of 595 nm. The protein concentration was calculated using the calibration curve of the protein standard curve of bovine serum albumin (BSA). The results were expressed in µg of microbial protein/mL. Where 0.6 % v/v sodium hypochlorite and sterile NSS served as positive and growth controls, respectively. Individual experiments were replicated three times.

Phenol coefficient test of ozone treated water

The phenol coefficient was applying to test the ozone-treated water according to previous report [6]. Briefly, the stock solution (5 % w/v) of phenol was prepared. Then, serial two- fold dilutions of various concentrations of phenol or ozone-treated water were performed by sterile distilled water, so that the concentrations ranged from 5-0.3125% w/v and 1:2-1:64 respectively. An isolated colony of each indicator microorganisms isolate (*Bacillus subtilis / Salmonella typhi*) was suspended in a sterile 0.85% NaCl solution, and the turbidity was adjusted equivalent to 0.5 McFarland standards corresponding to 1.5×10^8 CFU/ml. The microbial suspension was subsequently diluted to 10^4cell/ml. To assess the phenol coefficient, 700 µl of suspension containing each tested microorganism was added to individual tubes containing serial dilutions of phenol or ozone-treated water at a 1:1 ratio. After 5 and 10 minutes, 100 µl of the mixture from each different dilution was evenly spread onto Tryptic Soy Agar (TSA) plates and incubated at 37°C for 24 hours. Following the incubation period, bacterial growth was observed, and the Rideal-Walker Coefficient was calculated by identifying the highest dilution of the disinfectant that effectively killed microorganisms in 5 minutes but not in 10 minutes.

Comparison of efficacy of ozone-treated water with different disinfectants

The effectiveness of ozone-treated water was compared to other disinfectant solutions such as 0.6% v/v sodium hypochlorite,

potassium permanganate (0.05%w/v), baking soda mixture (2.5 g/L), and chlorinated water (0.02% w/v). Briefly, 100 ul of tested pathogenic microbial suspension (1.5 x 10^5 CFU/ml) was dropped onto the surface of a sterile 5x 5 cm plastic sheet and allowed to dry for approximately 1 hour. Then, 100 ul of various disinfectants were applied to the surface of the plastic sheet at different time points determined by the previous experiment (15 min, 20 min). After soaking the plastic sheet in a 1 mL sterile 0.85% saline solution tube, it was shaken for 1 minute to allow the saline solution to remove bacteria from the plastic. Then, 100 µl of the mixer was spread onto the TSA and incubated at 37°C for 24 hours. After an incubation period, the colony on the culture medium were manually counted and compared to the growth control (sterile NSS). The bacterial cells were presented as colony forming units (CFUs). Individual experiments were replicated three times.

Comparison of the efficacy of ozone-treated water by sterilization contact techniques and surface type

The effectiveness of sterilization contact techniques (immersion and spray) and surface type (plastic sheet and fabric clothes) by ozone water was evaluated. Briefly, 100 ul of tested pathogenic microbial suspension (~ 1.5 x 10^5 CFU/ml) was dropped onto the surface of difference sterile object (plastic sheet and fabric clothes) and leave to dry for about 1 hour. Then, the various contact techniques were performed as describe:

Immersion: Each object was immersed in 15 mL of ozone-treated water at various times (15 min, 20 min). The object was then placed in a 1 mL sterile 0.85 % saline solution tube and shaken for 1 minute to allow the saline solution to remove the microbial.

Spray: The surface of the different object was sprayed with ozone-treated water for 10 seconds (15 mL), and afterwards the object was left at a different time point (15 min, 20 min). The object was then immersed in a 1 mL tube of sterile 0.85% saline solution and shaken for 1 minute to allow the saline solution to remove microbial from the object.

And after that, 100 µl of the mixer was spread onto the TSA and incubated for 24 hours at 37°C. Following incubation, the colony counts on the culture medium were manually counted and compared to the growth control (sterile NSS) and expressed as colony forming units (CFUs). The experiment was done in triplicate.

Statistical analysis

Each experiment was performed in triplicate and results were expressed as mean ± SD. Data were evaluated by One-way analysis of variance (ANOVA) using SPSS (version 22.0) for significance ($p \leq 0.05$) and the Tukey test at the 95% confidence level.

RESULTSAND DISCUSSION

Ozone-treated water chemistry test

The ozone-treated water had a pH of 7.81±0.07 indicating that its properties were highly alkaline but less as compared to 0.6% NaClO with a very alkaline (pH 12.18±0.05). While distilled water was neutral (pH 7.20±0.05) (Table 1).

ORP measurement revealed that ozone-treated water had an ORP of -46.29 ± 0.06 mV, indicating that it is a medium oxidizing agent, while 0.6% Sodium hypochlorite and distilled water had an ORP of -309.67 ± 1.15 and -11.43 ± 0.58 mV, respectively as shown in Table 1.

Table 1: Chemical properties of sodium hypochlorite (0.6% NaClO), ozone water, and distilled water

Tested substance	pH	ORP (mv)
Ozone water	7.81± 0.07	- 46.29 ± 0.07
0.6% NaClO	12.18 ± 0.05	- 309.67 ± 1.15
Distilled water	.72 0± .005	- 11.43 ± 0.58

Table 2 The bactericidal activity test results of 0.6% NaClO (Positive control), ozone spray water, and ozone-treated water were shown in mean ± SD from the three identical tests.

Time		Type of disinfectant	Tested organism			
			Pseudomonas aeruginosa	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*
		Growth control	**4.70 ±0.04**	**5.06±0.04**	**4.72±0.04**	**4.45±0.08**
Viable count (Log CFU/mL)	1min	0.6% NaClO	0.00*	0.00*	1.30±0.00*	0.00*
		ozone spray water	3.72±0.12*	4.97±0.01*	3.26±0.24*	3.42±0.10*
		ozone treated water	2.55±0.23*	1.60±0.28*	2.78±0.11*	2.79±0.15*
	3 min	0.6% NaClO	0.00*	0.00*	0.00*	0.00*
		ozone spray water	0.00*	4.91±0.03*	0.00*	3.20±0.17*
		ozone treated water	2.26±0.60*	1.56±0.24*	2.62±0.23*	2.51±0.32*
	5 min	0.6% NaClO	0.00*	0.00*	0.00*	0.00*
		ozone spray water	0.00*	0.00*	0.00*	1.20±0.14*
		ozone treated water	2.18±0.24*	1.76±0.15*	0.43±0.58*	1.46±0.28*
	10 min	0.6% NaClO	0.00*	0.00*	0.00*	0.00*
		ozone spray water	0.00*	0.00*	0.00*	0.00*
		ozone treated water	1.94±0.30*	1.56±0.24*	0.21±0.13*	1.12±0.10*
	15 min	0.6% NaClO	0.00*	0.00*	0.00*	0.00*
		ozone spray water	0.00*	0.00*	0.00*	0.00*
		ozone treated water	1.20±0.89*	0.43±0.58*	0.00*	1.03±0.89*
	20 min	0.6% NaClO	0.00*	0.00*	0.00*	0.00*
		ozone spray water	0.00*	0.00*	0.00*	0.00*
		ozone treated water	1.04±0.17*	1.19±0.85*	0.00*	0.90±0.17*

(*) means a statistically significant reduction in the amount of bacteria (P<0.05).

Determination of bactericidal activity of ozone-sprayed water and ozone treated water

Both ozone-sprayed water and ozone-treated water were tested for their ability to inhibit the growth of clinically important pathogenic microorganisms in comparison to standard disinfectants. The amount of bacterial growth control was found to be in the range of log 4.45±0.08 – log 5.06 ±0.04. It was discovered that ozone-sprayed water kills both gram positive and gram negative bacteria. The amount of four test pathogen strains was significantly reduced after the first minute, and all organisms were killed within 1-5 minutes of exposure. However, ozone-treated water killed within 15-20 minutes of exposure. Table 2 shows that the standard disinfectant (The amount of four test pathogen strains was significantly reduced after the first minute, and all organisms were killed within 1-5 minutes of exposure. However, ozone-treated water killed within 15-20 minutes of exposure. The standard disinfectant (0.6% NaClO) was also capable of killing all tested microorganisms in one minute.) was also capable of killing all tested microorganisms in one-minute as shows in Table 2.

Bactericidal activity of ozone treated air

The ability of ozone treated air to inhibit the growth of clinically important pathogenic microorganisms was tested. The amount of bacteria growth control was found to be between log 4.32±0.06– log 4.55 ±0.01. It was discovered that ozone treated air kills gram positive and gram negative bacteria alike. As shown in Table 3, the number of four test pathogen strains was significantly reduced after the first minute, and all organisms were killed within 3-5 minutes of exposure.

Table 3: The bactericidal activity test results of ozone treated air were shown in mean ± SD from the three identical tests.

Tested organism	Growth control	Viable count (Log CFU/mL)					
		1min	3 min	5 min	10 min	15 min	20 min
Pseudomonas aeruginosa	**4.48±0.01**	**3.88±0.03***	**3.77±0.07***	**0.00***	**0.00***	**0.00***	**0.00***
Escherichia coli	**4.55±0.01**	**4.11±0.13**	**0.00***	**0.00***	**0.00***	**0.00***	**0.00***
Staphylococcus aureus	**4.32±0.06**	**3.89±0.11**	**3.62±0.24***	**0.00***	**0.00***	**0.00***	**0.00***
Bacillus subtilis	**4.53±0.03**	**4.38±0.07**	**4.09±0.09***	**3.65±0.33***	**3.49±0.20***	**3.20±0.17**	**0.00***

(*) means a statistically significant reduction in the amount of bacteria (P<0.05).

Cytotoxicity of ozone-treated water to OUMS-36 fibroblasts

The cytotoxicity experiment revealed that ozone-treated water at a concentration of 0.1-100 % v/v of ozone-treated water has a relatively low cytotoxic effect on OUMS-36 fibroblasts cells, with an IC50±SD value of 64.47 ± 4.45 ug/100 uL, as shown in Figure 1.

Intracellular protein leaking by dye-binding method (Bradford)

Intracellular protein leaking measures [7] the protein that leaks from the breaks down microbial cell. It was found that after the ozone-sprayed water was exposed to the microorganisms at different intervals, proteins from the intracellular organisms of the tested microorganisms were leaked from 1 minute after ozone-sprayed water exposure and relatively stable over the following periods at 3 - 5 min, as shown in figure 2B. Whereas, intracellular protein leaking were leaked 1 minute after exposure to 0.6% NaClO and relatively stable over that time point (2A).

Phenol coefficient test of ozone treated water

Table 4 shows the results obtained when dilutions of ozone-treated water were tested. At 1: 2 dilutions, growth of *B. subtilis* and *S.Typhi* was recorded at 5 minutes, but not at 10 minutes contact times, thus giving a Rideal-Walker Coefficient of 0.125.

Table 4: Determination of Rideal-Walker coefficient for ozone-treated water and phenol using *Bacillus subtilis* / *Salmonella* typhi as test organism

Disinfectant	Dilution of disinfectant	Contact time with culture (Minutes)		Phenol coefficient	Contact time with culture (Minutes)		Phenol coefficient
		Bacillus subtilis			*Salmonella* typhi		
		5 min	10 min		5 min	10 min	
Ozone-treated water	**1:2**	+	-	**0.125**	+	-	**0.125**
	1:4	+	+		+	+	
	1:16	+	+		+	+	
	1:32	+	+		+	+	
	1:64	+	+		+	+	
Phenol	**2.5% w/v**	-	+		-	-	
	1.25% w/v	+	-		+	-	
	0.625% w/v	+	-		+	-	
	0.3125% w/v	+	-		+	+	
	0.15625% w/v	+	+		+	+	

Comparison of efficacy of ozone-treated water with different common household disinfectants

The amount of tested bacterial growth control was found to be between log 5.41±0.31– log 6.13±0.02. Ozone-treated water has antimicrobial properties against some tested microbial strains. CFU growth of *P. aeruginosa* and *E. coli* was reduced or stopped after 15 minutes of exposure to ozone-treated water but not for *S. aureus* and *B. subtilis*. Common household disinfectants, however, such as 0.2 % potassium permanganate, 0.025 % chlorine, and 0.5 % baking powder,

were unable to kill all tested microorganisms at 3-5 minutes as well. As a result, as shown in Table 5, ozone-treated water had a lower disinfectant efficiency than conventional household disinfectants.

Comparison of the efficiency of ozone-treated water in sterilization by various contact techniques

According to the results of the tested plastic sheet, the amount of tested bacterial growth control was in the range of log 5.29±0.13– log 6.02±0.02. Both immersion and sprayed techniques were found to kill most pathogens tested within 15-20 minutes after exposure except for *B. subtilis*. The results of the tested cloth pads revealed that the amount of tested bacterial growth control was between log 5.34 ± 0.05 – log 6.24±0.05. Both immersion and sprayed techniques were found to be capable of killing two tested pathogens within 15 minutes of exposure except *S. aureus* and *B. subtilis*. Whereas the standard disinfectant (0.6% NaClO) able to kill all of the tested pathogens from the fabric within 5 minutes in all technique, shown in Table 6.

Figure 1: Cytotoxicity results of the OUMS-36 fibroblasts of ozone treated water.

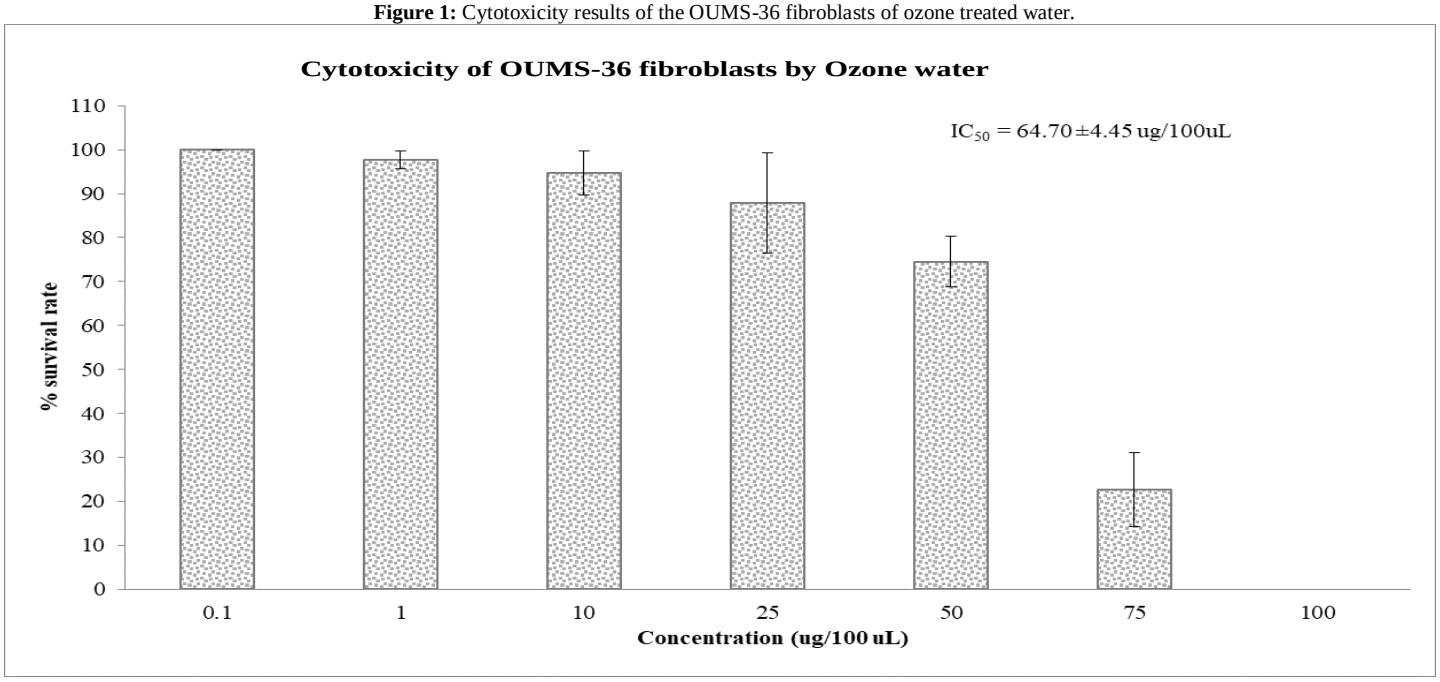
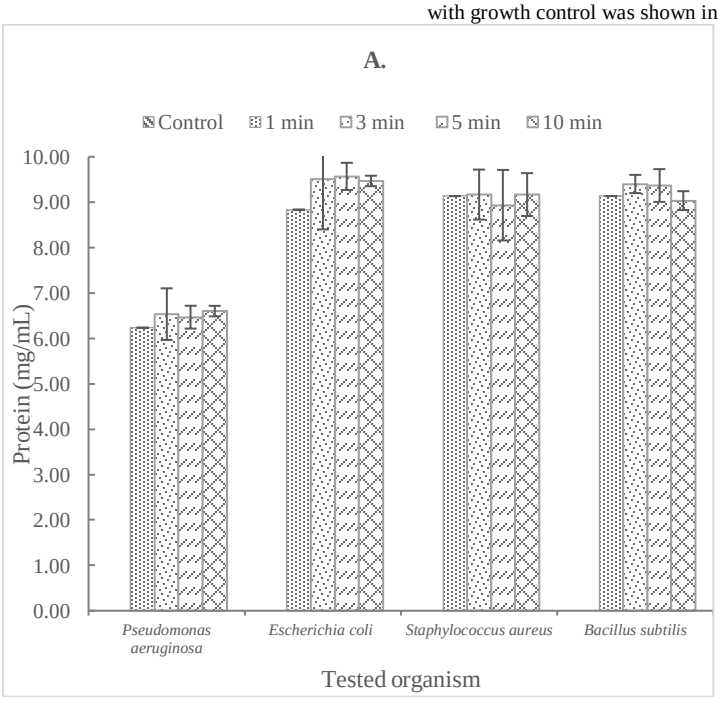


Figure 2: The amount of protein released from tested microbial cells destroyed with A.0.6% Sodium hypochlorite and B. ozone spray water at different times compared with growth control was shown in mean ± SD from the triplicate test.



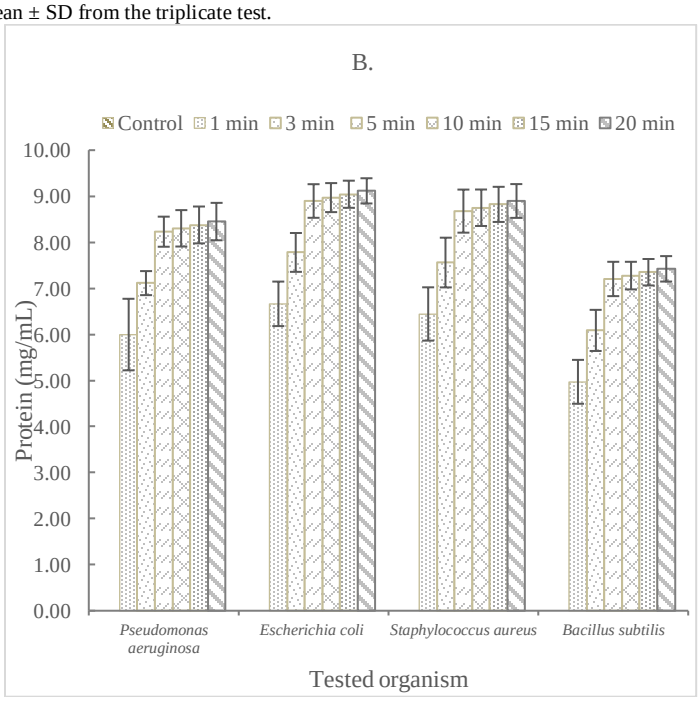


Table 5: Comparison of the efficacy of ozone-treated water with different disinfectants. The values were shown in mean±SD from the three identical tests.

Tested organism	Viable count (Log CFU/mL)										
	Growth control	0.6% NaClO		ozone treated water		0.2% potassium permanganate		0.025% chlorine		0.5% baking powder	
		3 min	5 min	15 min	20 min	3 min	5 min	3 min	5 min	3 min	5 min
Pseudomonas aeruginosa	5.41±0.31	0.00*	0.00*	4.49±0.20*	4.49±0.20*	0.00*	0.00*	0.00*	0.00*	4.69±0.09	0.00*
Escherichia coli	5.99±0.04	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	5.14±0.12	5.14±0.12	5.49±0.05	5.04±0.12
Staphylococcus aureus	5.96±0.03	0.00*	0.00*	4.54±0.28*	4.54±0.28*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
Bacillus subtilis	6.06±0.02	0.00*	5.60±0.07*	4.73±0.15*	4.73±0.15*	4.49±0.20*	4.74±0.13*	5.11±0.12*	5.11±0.12*	5.67±0.07*	5.51±0.06*

(*) means a statistically significant reduction in the amount of bacteria (P<0.05).

Table 6: Comparative of the efficacy results from ozone-treated water for sterilization on plastic sheets or cloth pad by immersion or spray technique were shown in mean ± SD from 3 identical tests.

Tested organism	Viable count (Log CFU/mL)																	
	plastic sheets									cloth pad								
	Growth control	Immersion				Spray				Growth control	Immersion				Spray			
		0.6% NaClO		ozone treated water		0.6% NaClO		ozone treated water			0.6% NaClO		ozone treated water		0.6% NaClO		ozone treated water	
		3 min	5 min	15 min	20 min	3 min	5 min	15 min	20 min		3 min	5 min	15 min	20 min	3 min	5 min	15 min	20 min
Pseudomonas aeruginosa	5.29±0.13	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	5.48±0.02	0.00*	0.00*	4.43±2.08*	0.00*	0.00*	0.00*	0.00*	0.00*
Escherichia coli	5.40±0.01	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	5.34±0.05	0.00*	0.00*	4.43±2.08*	0.00*	0.00*	0.00*	0.00*	0.00*
Staphylococcus aureus	5.68±0.03	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	6.29±0.01	0.00*	0.00*	4.63±1.15*	4.10±0.17*	0.00*	0.00*	2.67±0.58*	4.10±0.17*
Bacillus subtilis	5.88±0.02	0.00*	0.00*	4.70±0.09*	4.10±0.17*	0.00*	0.00*	5.41±0.07*	5.04±0.04*	5.88±0.02	0.00*	0.00*	5.33±8.74*	4.76±0.15*	0.00*	0.00*	5.33±8.74*	4.76±0.15*

(*) means a statistically significant reduction in the amount of bacteria (P<0.05)

DISCUSSION

Effective disinfectants play a crucial role in combating the spread of microorganisms. Ideally, a disinfectant should possess a broad-spectrum activity, targeting a wide range of microorganisms rather than being specific. Furthermore, it should be versatile enough to be applied on various surfaces and objects, inanimate ones, to prevent microbial transmission. Various mechanisms underlie the action of disinfectants against microorganisms. These mechanisms include cell rupture or leakage, disruption of intracellular balance, interference with cell membrane function, inhibition of enzyme activity, disruption of electron transfer processes, among others [8].

In contemporary disinfection practices, chemical disinfectants are widely utilized. Among them, chlorine-containing compounds like sodium hypochlorite (NaClO) are commonly employed. Sodium hypochlorite dissolves in water, forming hypochlorous acid (HOCl). This reactive substance interacts with microbial proteins and oxidizes organic molecules, thereby exerting its disinfecting effect. However, it's important to note that these chemicals can pose direct health risks to human skin and mucous membranes. They can lead to respiratory issues, tissue and skin irritation, and even corrode certain metals [9,10]. Consequently, they are not suitable for applications where direct contact with humans or consumption is a concern. Considering the side effects and health risks associated with traditional chemical disinfectants, it is prudent to explore safer alternatives. One such option is the use of ozone generators.

Ozone generators have gained attention as a promising alternative to traditional chemical disinfectants due to their broad-spectrum antimicrobial activity and lack of harmful byproducts. Ozone (O3) is an unstable molecule with strong oxidative properties that can effectively eliminate a wide range of microorganisms, including bacteria and viruses [11]. Unlike some chemical disinfectants, ozone does not leave behind harmful residues and does not contribute to the development of resistant strains of microorganisms [12]. Moreover, ozone can be generated on-site, providing a sustainable and cost-effective solution for disinfection needs. It can be applied in various settings, including healthcare facilities, food processing plants, and public spaces.

However, it is important to note that ozone is an unstable compound with a much shorter half-life in water than in air. The results of the ozone-treated water chemistry test showed that the ozone-treated water had a pH of 7.81±0.07, which has a neutral pH.

The oxidation reduction potential (ORP) is -46.29±0.07 which is lower than 0.6% sodium hypochlorite. It is worth noting that ozone is an unstable compound with a much shorter half-life in water than in air [13]. These findings underscore the effectiveness of ozone as a disinfectant, not only in terms of its antimicrobial properties but also its ability to maintain water quality with a neutral pH, making it a compelling choice for various applications where disinfection is required while avoiding the drawbacks associated with chemical alternatives.

To determine the minimum time for killing microorganisms, the contact time of an ozone generator was tested at 1, 3, 5, 10, 15, and 20 minutes. All organisms were killed within 1-5 minutes and 3-5 minutes of exposure to ozone-sprayed water and ozone-treated air, respectively. Ozone-treated water, on the other hand, killed within 15-20 minutes of exposure. This is consistent with the findings of previous studies that ozone-treated air and ozone-sprayed water can kill interested bacteria within the first 10-30 seconds of exposure [14,15]. The reason why ozone-treated water takes more time to sterilize than ozone-sprayed water and ozone-treated air may be due to O_3 has a half-life of 20-30 minutes in distilled water at 20 ˚C before converting back to an oxygen. molecule. However, gaseous O_3 is more stable in atmospheric air, with a half-life of about 12 hours [16].

The experiment was corresponding to the intracellular protein leaking results which show that the mechanism by which oxidative stress of O_3 destroys microbial cells influences the global polarity of the bacterial surface, involving lipid peroxidation and the degradation of trans membrane proteins that control ion flow. Thus, cells rupture, resulting in ion leakage between the media, resulting in microorganism cell rupture, protons leak out, and eventually death [17-18]. The bactericidal activity of this agent can also be identified by its direct action on the organic compounds of these microorganisms, affecting their metabolisms [19]. Before using ozonized water for human disinfection, it is critical to rule out the possibility of cytotoxic effects in mammalian cells. We tested this parameter and found it to be cytotoxic in this mammalian cell line. The cytotoxicity results of ozone-treated water as relatively low toxicity effect to OUMS-36 fibroblasts. It was assumed that ozone-treated water is safe and harmless to human tissue. Previous research has shown that some mammalian cells have complex antioxidant systems that prevent ozonolysis at the cytoplasmic membrane and resist the other oxidant properties of O_3, even when the gas is dissolved in water [20].

The efficacy of ozone-treated water was compared to various disinfectants commonly used in households. Ozone-treated water was found to have a lower disinfectant efficiency than conventional household disinfectants. Because the number of gram-positive pathogens tested was only reduced 15 minutes after exposure. However, common household disinfectants such as 0.2% potassium permanganate, 0.025% chlorine, and 0.5% baking powder also failed to kill all tested microorganisms after 3-5 minutes but less time of exposure.

The disinfection potency of ozone-treated water versus 0.6% NaClO on surfaces of transparent plastic and cloth contaminated with tested pathogens was compared using various contact techniques (immersion and spray). It was discovered that ozone-treated water sterilized the bacterial contamination on the plastic sheet by both immersion and spray technique within 15-20 minutes of exposure, except for *B. subtilis*. While sterilization tests on cloth pads of ozone-treated water using both immersion and spray techniques produced similar results, reducing 2 of 4 germs in 15 minutes with the exception of *S. aureus* and *B. subtilis*. However, 0.6% NaClO was found to be capable of killing all of the tested pathogens from the plastic sheet and fabric within 5 minutes. This finding was supported by Megahed and colleagues, who demonstrated that the killing capacity of O_3 exposure differed depending on the surface, with smooth surfaces having a significant impact on O_3 concentration [19].

Although a recent study found gram positive bacteria resistance to ozone-treated water, our current study found that using ozone-sprayed water and ozone-treated air had a higher efficacy in reducing bacterial contamination in a time-dependent manner. Given this and the current efficacy of ozone generators in many therapeutic and disinfecting procedures, ozone generators may be efficiently included in disinfection technologies.

While ozone generators offer promise as safer disinfection methods, it is essential to conduct further research and gather evidence to establish their effectiveness in specific applications. Moreover, safety protocols for ozone use need to be well-defined to ensure that potential health risks are minimized. Research and ongoing studies in this field are critical to providing robust scientific support for the adoption of ozone-based disinfection methods in various settings.

CONCLUSION

Given the relevant biocidal aspect of ozone demonstrated by our results, an ozone generator is a potential sanitizer not only for surface decontamination but also as an agent to reduce the spread of pathogenic microorganisms in the environment. Disinfecting time is 1-3 minutes after exposure. Immersion techniques and smooth surface object were found to be more effective for cleaning contaminated than spraying and fabrics, which take only 15-20 minutes to disinfect. As a result, because of its high biocompatibility, ozonized generators can be useful in cleaning surface devices, and could be used in the methodologies or devices to replace disinfectants considered toxic by regulatory agencies.

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Conflict of interest statement

The author declares that they have no conflict of interests.

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