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ORIGINAL RESEARCH

Analysis of Bactericidal Effect of Three Medical Ozonation Dosage Forms on Multidrug-Resistant Bacteria from Burn Patients

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Objective: To examine the bactericidal effects of three different states of medical ozone (liquid, gas, and oil) against drug-resistant strains of common bacteria on burn wounds, which could as a clinical reference.

Methods: Three multidrug-resistant strains of methicillin-resistant *Staphylococcus aureus*, pan-resistant *Pseudomonas aeruginosa*, and ESBLs *Klebsiella pneumoniae* were identified from burn wounds. The colonies of the three varieties of bacteria were each carried out using the pour plate method prior to the start of the experiment. Then, depending on the state of ozone, different treatment procedures are applied. Group of ozone gas: in a closed glass jar, the bacterial liquid was injected into a single layer of sterile gauze, and the ozone gas concentration was held at 50 g/mL. The bacterial liquid was diluted and combined directly with ozone water in the ozone water group. Ozone is a type of oil: after the emulsifier was added to the oil group. The gas, water, and oil groups were rapidly neutralized and counted again after 5, 10, and 30 minutes.

Results: Ozone gas and oil groups totally eliminated multidrug resistant bacteria in the above study within 30 minutes. (2) At 5 and 10 minutes, the difference in bactericidal effect between ozone gas group and ozone water and oil group was statistically significant ($P<0.05$), and there was no significant difference between ozone water and oil groups ($P>0.05$); at the time of 30 minutes, the effects of bactericidal effect between ozone water group and ozone gas and oil had no significance ($P> 0.05$).

Conclusion: Ozone has the ability to kill bacteria, depending on the treatment time, different ozone types should be chosen for sterilization and disinfection in clinical application.

Keywords: ozone, burn, wound treatment, multidrug resistant bacteria

Introduction

Burns are a serious and widespread problem. Large-area burns can not only cause direct skin tissue damage and internal environment disorders, but they can also cause severe bacterial infection due to wound exposure.[1] Infection and multiple organ failure caused by infection are important causes of death in burn patients.[2] With the increasing resistance of bacteria, searching for topical agents that have good bactericidal effects on widely resistant bacteria may be a significant way to control antimicrobial infection in burn patients. Consisting of three oxygen atoms, Ozone has a strong bactericidal effect with its extremely strong oxidation nature and is widely applied in the medical field.[3,4] Bacterial activity in ozone has been investigated since the 19th century. Ozone has a powerful antibacterial impact; even a small amount of ozone can destroy bacteria. Escherichia coli, Salmonella, Streptococcus, and other bacteria have all been discovered to be vulnerable to ozone inactivation. Ozone's antibacterial impact primarily affects bacteria's integrity by disrupting their membrane structure. Furthermore, ozone can be utilized to eliminate fungi

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or amoeba in a wound.[2,4,5] After disinfection, ozone can naturally decompose and restore into the oxygen molecular of two oxygen atoms, producing no residue and pollution, and avoids the resistance problem of antibiotics and the limitations of the use of conventional chemical disinfectants. At present, the clinical application forms of ozone mainly include gas, water and oil.[6,7] Three ozone states are used in clinical research, depending on the wound's status.[8–11] In order to compare the bactericidal effects of three medical ozone formulations on common pathogenic bacteria resistant strains of burn wounds, we treated simulated wound wounds with three different states of ozone to evaluate the killing effect of different states of ozone on fungi near burn wounds.

Materials and Methods

Experimental Materials Medical Ozone

Generator and ozone oil agent were provided by Wuhan Zhihong Biotechnology limited company. The neutralizing agent (pH=7.2) was 5.0 g/L sodium thiosulfate, 10 g/L lecophosphate, 10 g/L tewain-80 and was prepared using phosphate buffer. Hangzhou Tianhe Microbial Reagent limited company acquired Nutrition Aar Dry Powder and Petri dish. Guangzhou Dijing Microbial Technology limited company provided the completed product medium. The finished product medium was purchased in Guangzhou Dijing Microbial Technology limited company. PHOENIX100 automatic bacterial identification analyzer and turbidimeter were bought in BD. Deep cryrefrigerator and thermostat incubator are purchased at Thermo.

Strain Origin

The experimental strains were isolated from the culture of wound secretions in patients with the Severe Burn Unit (BICU) from June 2016 to July 2018 by the bacterial chamber of the Burn Research Institute of Wuhan Third Hospital. At least 40 multi-resistant strains resistant to 3 types of antibiotics were selected, including 15 methicillin-resistant *Staphylococcus aureus*, 13 pan-resistant *Pseudomonas aeruginosa* and 12 *Klebsiella pneumoniae* with ESBLs. The experimental strains were preserved at −80°C using the slip method after purification. The above strains were revived when tested, and 3~5 strains were randomly selected and dissolved in 5 mL 0.9% sodium chloride solution. We corrected the fluid concentration to 3.0108 colony-forming unit/ microliter (cfu /mL) with turbidimeter. The turbidity is equivalent to 1.0 MCF.

Intervention Method and Group Processing

According to the three forms of ozone, we divided the experimental strains into ozone gas group, ozone water group and ozone oil group. The study was approved by the ethics Committee of our hospital, and the patients agreed to collect samples for the study.

Ozone Gas Group

We applied 1 mL bacteria solution to a single sterile gauze sheet in the flat dish. We connected the ozone generator to the ozone bottle and the pipe port, and inserted the ventilation pipe into the closed glass tank, adding a small amount of distilled water at the tank bottom to keep the relative humidity in the tank 50% −70% and the ozone concentration in the tank 50 μg/mL. The gauze plates containing the strain were placed into the tank and the lids were uncovered. We kept the gauze plates standing still at 5, 10, 30 minutes respectively and turned off the ozone generator gas switch at the corresponding time. We took the flat dishes out from the glass tank, placed the gauze sheet in the flat dishes into the test tubes containing neutralizing and mixed for 5 min. After gradient dilution, we added nutrient agar into the tubes and placed the tubes in a 35°C incubator for 24 h for live bacteria count.

Ozone Water Group and Ozone Oil Group

Referring to the method from literature,[12,13] we combined 0.5 mL of bacteria solution with 4.5 mL of ozone water after diluting the primary bacterium solution to 3.0×105 cfu/mL. The ozone oil group was a mixture of ozone oil and emulsifier for 4.5 mL (with an ozone oil content of 60 g/L). After standing 5, 10, 30 min, we added 0.5 mL of the mixture to the test tube with 4.5mL neutralizing agent for 5 minutes, and then adopted gradient dilution, poured nutrient agar in the tube, and culture for 24 h in a 35 ° incubator for live bacteria count.

Bactericide Effect Calculation

After culturing for 24 h, we took out the flat dishes, counted the colonies, and made a comparison with the amount of primary liquid bacteria. The average bactericide rate was calculated.:

Bactericide rate formula= (NBB-NBA)/NBB× 100%.

NBB: Number of bacteria of primary bacteria solution on the flat dishes before intervention (cfu/L); NBA: Number of bacteria after intervention (cfu/L).

If there was no colony on the flat dishes, bactericide rate was 100%.

Statistical Methods

The resulting data were statistically analyzed using the spss 20.0 software. The measurement data is represented by x ± s. Single-factor variance analysis was used among multiple groups. LSD-*t* test was adopted between two groups. The variance analysis between multiple time points used repeated measurement variance analysis. Test for sphericity and within-subject effects with the Mauchly test. The spherical test's outcome determines whether we are dealing with a multivariate or within-subjects test. If the spherical test yields a $p<0.05$, the spherical assumption is violated, and the multivariate test is required. The multivariate test will offer four techniques, the first of which is the most reliable. When the results of the four approaches disagree, the first method takes precedence. If the spherical test $p > 0.05$, it follows the spherical assumption and can look at the within-subject effect test directly.[14,15] $P <0.05$ is statistically significant for the differences.

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Result

The Bactericidal Effect of 3 Types of Ozone

The bactericidal effect of 3 types of ozone on drug-resistant strains of common pathogenic bacteria in burn wounds is shown in Table 1. The bactericidal rate remained above 95% at 30min in the the ozone gas group and at 5, 10, and 30min in the ozone water and ozone oil groups. At the 5 and 10min, the bactericidal rate of the ozone group was significantly lower than that of the ozone water and oil groups, and the differences were statistically significant ($P <0.05$). There is no statistical difference in the bactericidal rate between the ozone water group and the ozone oil group at the 5 and 10min ($P> 0.05$). At 30min, the bactericidal rate of the ozone water group was significantly lower than that of the ozone gas group and the ozone oil group, and the differences were not significant ($P> 0.05$). There is no statistical difference in the bactericidal rate between ozone gas and ozone oil at 30min ($P> 0.05$).

Interaction of Processing Grouped by Types and Intervention Time

The groups take time as subjective effect. The results showed that the indicators between different time points in the *Staphylococcus aureus* group did not meet the spherical test ($P <0.05$), and the degree of freedom was corrected during repeated measurement analysis. The indicators in the Pseudomonas and *Klebsiella pneumoniae* group met spherical tests ($P> 0.05$). Correction degree of freedom is not required for repeated measurement analysis (See Table 2).

The difference analysis of the bactericidal effects of the three types of ozone at multiple time points can adopt repeated measurement variance analysis, showing a statistical significance among different time points ($P <0.05$). There are statistically significant differences across groups ($P <0.05$). There was also statistical significance in the interaction between different time points and grouping ($P <0.05$) (See Table 3).

Discussion

Patients with burns and other skin abnormalities are at risk of infection due to the loss of the skin barrier. Infection and infection-related multiple organ failure are the first cause of burn death. Early prevention and control of infection is the

Table 1 Comparison of the Bactericidal Ability of 3 Types of Ozone Towards Common Bacteria in Burn Wounds at Different Time Points

Group			Ozone Gas	Ozone Water	Ozone Oil	F value	P value
Staphylococcus aureus (n=15)	5min	Number of living bacteria (cfu)	1.52±0.06^{a,**}	0.10±0.02	0.11±0.03^{b,**}	5837.537	<0.001
		Bactericidal rate (%)	49.41±1.96	96.74±0.66	96.21±1.02	–	–
	10min	Number of living bacteria (cfu)	1.13±0.08$^{a,\Delta\Delta}$	0.01±0.01	0.00±0.01$^{b,\Delta\Delta}$	3271.379	<0.001
		Bactericidal rate (%)	62.39±2.42	99.56±0.26	99.91±0.18	–	–
	30min	Number of living bacteria (cfu)	0.00±0.00$^{a,\#\#}$	0.01±0.01$^{c,\#\#}$	0.00±0.00	1.906	>0.05
		Bactericidal rate (%)	100	99.72±0.24	100	–	–
Pseudomonas aeruginosa (n=13)	5min	Number of living bacteria (cfu)	1.01±0.07^{a,**}	0.13±0.02	0.12±0.03^{b,**}	1699.335	<0.001
		Bactericidal rate (%)	66.26±2.17	95.64±0.67	95.93±0.99	–	–
	10min	Number of living bacteria (cfu)	0.61±0.07$^{a,\Delta\Delta}$	0.02±0.02	0.00±0.01$^{b,\Delta\Delta}$	801.400	<0.001
		Bactericidal rate (%)	79.66±2.39	99.64±0.23	99.87±0.21	–	–
	30min	Number of living bacteria (cfu)	0.00±0.00$^{a,\#\#}$	0.01±0.01$^{c,\#\#}$	0.00±0.00	1.044	>0.05
		Bactericidal rate (%)	100	99.59±0.44	100	–	–
Klebsiella pneumoniae (n=12)	5min	Number of living bacteria (cfu)	1.32±0.05^{a,**}	0.09±0.03	0.08±0.03^{b,**}	3890.445	<0.001
		Bactericidal rate (%)	55.96±1.70	96.77±0.79	97.25±1.09	–	–
	10min	Number of living bacteria (cfu)	0.96±0.08$^{a,\Delta\Delta}$	0.01±0.01	0.00±0.00$^{b,\Delta\Delta}$	1519.401	<0.001
		Bactericidal rate (%)	68.01±2.61	99.55±0.36	99.95±0.11	–	–
	30min	Number of living bacteria (cfu)	0.00±0.00$^{a,\#\#}$	0.01±0.01$^{c,\#\#}$	0.00±0.00	0.853	>0.05
		Bactericidal rate (%)	100	99.76±0.27	100	–	–

Notes: ^{a}Ozone gas vs ozone water, ^{b}Ozone gas vs ozone oil, ^{c}Ozone water vs ozone oil. Compared with this group at 5 min $^{**}P<0.01$; compared with this group at 10 min: $^{\Delta\Delta}P<0.01$; compared with this group at 30min: $^{\#\#}P<0.01$. cfu (colony-forming unit): it estimates the number of bacteria or fungal cells in a sample which are viable, able to multiply via binary fission under the controlled conditions. F-value: the ratio of two variances, or technically, two mean squares. Mean squares are simply variances that account for the degrees of freedom (DF) used to estimate the variance. F-values are the test statistic for F-tests. Learn more about test statistics. p-value: a p-value is a measure of the probability that an observed difference could have occurred just by random chance.

Table 2 Spherical Test Results of Bactericidal Effects of Three Types of Ozone at Different Time Points

Group	Mauchly's W	The χ^2 Value That Was Last Read	Degree of Freedom	P value
Staphylococcus aureus (n=15)	0.687	15.369	2.000	<0.001
Pseudomonas aeruginosa (n=13)	0.997	0.102	2.000	0.95
Klebsiella pneumoniae (n=12)	0.893	3.618	2.000	0.164

Notes: Mauchly's W: Mauchly's sphericity test. The χ^2 value that was last read: a chi-square (χ^2) statistic is a measure of the difference between the observed and expected frequencies of the outcomes of a set of events or variables. Chi-square is useful for analyzing such differences in categorical variables, especially those nominal in nature. Degree of freedom: degrees of freedom refers to the maximum number of logically independent values, which are values that have the freedom to vary, in the data sample.

key to the success of clinical treatment. In recent years, the common wound infections of burn patients in China mainly include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* and so on. The bacterial resistance to antibiotics increased year by year,[16,17] posing significant issues for clinical treatment and in-hospital infection control. Pathogenic microorganisms from a burn wound can infiltrate deep wounds and living tissue, resulting in sepsis or sepsis with blood infection. For the prevention and treatment of burn infection, as well as the management

Table 3 Interaction of Group Processing and Different Intervention Times of 3 Types of Ozone

Group	Variables	Type III Sum of Squares	Degree of Freedom	Mean Square	F value	P value
Staphylococcus aureus (n=15)	Time	7.654	1.524	5.023	3042.870	<0.001
	Time*group	11.187	3.047	3.671	2223.833	<0.001
	Group	21.304	2.000	10.652	9431.354	<0.001
Pseudomonas aeruginosa (n=13)	Time	3.401	2.000	1.701	1625.928	<0.001
	Time*group	3.597	4.000	0.899	8 59.877	<0.001
	Group	6.331	2.000	3.166	1645.882	<0.001
Klebsiella pneumoniae (n=12)	Time	4.579	2.000	2.290	2065.026	<0.001
	Time*group	6.716	4.000	1.679	1514.331	<0.001
	Group	12.673	2.000	6.337	3625.360	<0.001

Notes: Type III sum of squares: the type III sums of squares are also called partial sums of squares. The type III sums of squares are not sequential, so the order of specification does not matter. Besides, the type III sums of squares do specify an interaction effect. Mean square: is a gauge of the average amount of variation explained by extraneous variables (the unsystematic variation). Time * group = interaction between group and time.

of severe burn wards, finding a safe and effective topical bactericide that is easy to apply and has fewer side effects is critical.

Ozone has the characteristics of oxidizing organic compounds, can act directly on the cell membrane of bacteria, and reacts with the double bond of lipids, damaging the composition of the cell wall and cell membrane and making the intracellular components degeneration, dissolution, and death.[17] Ozone is widely used in many aspects of medicine.[3,18]

Ozone has a strong bactericidal effect of on a variety of microorganisms.[19] The bactericidal effect of ozone gas, water, and oil on three strains of multiple drug-resistant pathogens with the highest detection frequency of burn wounds in recent years was observed in vitro in this study. The findings showed that the three types of ozone had obvious bactericidal effect. But the onset time and effects were different. Comparison results of the interaction analysis of grouping and time showed significant differences. The bactericidal effect of the ozone gas group at 5 and 10 min was significantly lower than that of the ozone water and ozone oil group ($P < 0.01$). Given the bactericidal ability of ozone gas is closely related to air humidity, environmental temperature and other reasons[20] and ozone gas surface disinfection ability is poor,[21,22] this study used the quantitative bacterial solution inoculation with gauze tablets, to some extent increasing the local humidity and simulating the conditions of a burn wound or a chronic refractory wound with an ozone bath. There was no significant difference in the bactericidal effect between the ozone water group and the ozone oil group at 5 and 10 min ($P > 0.05$), both reaching above 95% at 5 min. In a short time (5, 10 min), the bactericidal effect of the ozone water group and the ozone oil groups was better than that of the ozone gas group ($P < 0.01$). The ozone water group was less bactericidal than the ozone gas and ozone oil groups at 30 min ($P < 0.01$). Compared with 10 min, the bactericidal effect of the ozone water group at 30 min was not significantly improved, and there are still residual pathogenic bacteria, probably due to the continuous degradation of the ozone in the water and the failure to maintain the effective bactericidal amount at 30 min.

Ozone breaks down fastest in air, and dissolution in water is secondary. Neither of these types will suffer from residual or secondary contamination. During use, ozone flows with air and liquid and can act evenly on places not prone to conventional medical change. After the ozone is dissolved in the vegetable oil, it forms a stable peroxide derivative with the organic matter in the vegetable oil, while maintaining a stable strong oxidation capacity.[23] The three types of ozone are used in wound restoration with their own strengths.[17,24] But in our practical application, we also find some disadvantages. For example, the rate of ozone degradation in ozone water is far too rapid. Furthermore, controlling and maintaining the ozone concentration in the ozone bath is difficult. The stimulation of ozone water to wash the wound is obvious, and it is difficult for the ozone oil to fully attach to the mucosa and suppurative wound. Studies have also shown that ozone treatment requires window ventilation and suspends the use of antioxidant supplements, because they can affect the effect of ozone as an oxidant. Improper inhalation of ozone has an effect on the lungs which is very sensitive to oxidative damage caused by ozone due to the anatomy and biochemical properties of lung.[25] Therefore, the role of ozone in hospital infection control and body tissue repair remains to be further studied.

Conclusion

1) Ozone has the ability to kill bacteria that mimic wounds. 2) There was no significant difference in the bactericidal effect of the ozone water group and the odorous oil group in a short period of time, and they were all better than the ozone gas group; 3) Depending on the treatment time, different ozone types should be chosen for sterilization and disinfection in clinical application.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Wuhan Wudong Hospital, and informed consent from the patient was obtained.

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Disclosure

All of the authors had no any personal, financial, commercial, or academic conflicts of interest in this work.

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