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Bactericidal Activity and Physiological Effects of Combined Application of Poly-Hexamethylene Biguanide Hydrochloride and 1-bromo-3-chloro-5, 5-dimethylimidazolidine-2, 4-dione on *Staphylococcus aureus*

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***Abstract*—Poly-hexamethylene biguanide hydrochloride (PHMB) and 1-bromo-3- chloro-5,5-dimethylimidazolidine-2,4-dione (BCDMH) are widely used for surface disinfection. Here, we examined the bactericidal effects of the mixture of PHMB and BCDMH (named PB) combined in different ratios on *S. aureus*, and studied effects of temperature, pH and bovine serum albumin (BSA , as organic interferent) on PB bactericidal activity with suspension quantitative germicidal method. We also observed surface structure changes and ATP level in *S. aureus* treated with PB with scanning electron microscope and multi-function microplate reader, respectively. The results showed that mixture of 10 mg/L PHMB and 10 mg/L BCDMH could meet the bactericidal requirements according to Technical Standard for Disinfection (China 2002), producing similar bactericidal activity to those exhibited by 320 mg/L PHMB and 60 mg/L BCDMH, separately. The results showed that PB was competent to kill bacteria quickly and bactericidal activity increased with time within 12 min. Temperature had no significant effect on PB bactericidal activity. PB offered satisfied bactericidal activity within 3.0 to 5.0 pH range. BSA significantly reduced bactericidal activity of PB. In addition, PB treatment could result in protuberance on *S. aureus* surface, and bring about a rapid decline in intracellular ATP level.**

Keywords-poly-hexamethylene biguanide hydrochloride; 1-bromo-3-chloro-5, 5-dimethylimidazolidine-2, 4-dione; staphylococcus aureus; bactericidal activity; physiological effect

I. INTRODUCTION

Poly-hexamethylene biguanide hydrochloride (PHMB) has broad applications in water treatment, mouth wash formulations, antifungal remedies, and treatment of infective keratitis caused by *Acanthamoeba*. It is also used for beer glass sanitization and solid-surface disinfection [1-3]. PHMB exhibits bacteriostatic at low concentrations (10 mg/L). It can slightly damage the cell membrane, interact with bacterial DNA, and inhibit bacterial proliferation [2, 4]. No one resistant microorganism mutant has been reported after several decades of extensive use [5]. It is non-toxic to mammalian cells and relatively safe for human. Nevertheless, the high price and high working concentration restrict its broader application.

Another disinfectant, 1-bromo-3-chloro-5,5-dimethylimidazolidine-2,4-dione (BCDMH), is commonly used for swimming water treatment and environmental disinfection. When dissolved in water, it produces HClO and HBrO, which can oxidize a variety of proteins in microorganisms, especially enzymes. BCDMH has a strong bactericidal effect at low concentrations and is inexpensive. BCDMH has its own shortcoming, such as strong irritant smell and high risk of contact dermatitis [6].

To date, no study on combined use of PHMB and BCDMH has been reported. Because these two disinfectants work in different mechanisms, combined use may complement each other's drawbacks, and act synergistically. If yes, it is probable to reduce the minimal effective dose and overcome their side effects. Therefore, in the present study, *S. aureus* cells were treated with PHMB and BCDMH mixed in different ratios, and the effects of common factors such as temperature, pH and organic interferents (BSA used in our experiment) on bactericidal activity were studied. The effects of these biocides on bacterial surface structure and intracellular ATP content were also studied.

[1]Contributed equally to the work

II. MATERIALS AND METHODS

A. Microorganisms, Reagents, and Equipment

S. aureus ATCC 6538 was offered by Entry-Exit Inspection and Quarantine Bureau (Shanghai, China). The third generation of bacterial cultures were obtained according the procedure described in Technical Standard for Disinfection, China 2002, and stored at 4 ℃.

The field emission scanning electron microscope (SU 8010) we used was from Hitachi (Tokyo, Japan). Multi-function microplate reader was from PerkinElmer (USA). BacTiter-Glo™ Microbial Cell Viability Assay was purchased from Promega (USA). PHMB and BCDMH were purchased from Hangzhou Luochuan Chemical Co., Ltd., and Taixing Jiansheng Fine Biological Technology Co., Ltd., respectively.

B. Quantitative Suspension Test

Mix 1mL resulting cell suspension prepared in section 2.1 with 4 mL solution containing PHMB, BCDMH, or both (i.e., PB) and incubated for different time periods (10 min for PHMB, and 3 min for BCDMH and PB). The following procedures were conducted as quantitative suspension test described in Technical Standard for Disinfection, China 2002 .

C. Time, pH, Temperature and Organic Interferent Test

Reaction time: Take 0.5 mL of the mixture in 3 min, 6 min, 9 min and 12 min and mixed with the neutralizer respectively after the bacterial suspension was mixed with the disinfectant. Add 1 mL of the mixture to a sterile dish and pour into melted TSA, after coagulation, and incubate them for 48 h at 37 ° C in a incubator. The number of colonies was counted and KL was calculated.

pH: The pH of PB solution was adjusted to 3.0, 5.0, 7.0, 9.0, 11.0 respectively by 1mol / L sodium hydroxide or 1mol / L hydrochloric acid.

Temperature: temperatures were set to 0 ℃, 10 ℃, 20 ℃, 30 ℃, and 40 ℃.

Organic interferent: BSA was added to bacterial suspension first and mixed with disinfectant. There final concentrations of BSA, 0.3%, 0.6%, 0.9%, were set respectively.

D. Scanning Electron Microscopy

Cell suspensions were mixed with PB or sterile hard water solutions and then neutralized and incubated as described in section 2.2. The solutions were centrifuged at 1077 × g for 15 min and washed twice with PBS. The precipitates were resuspended in 100 μL of PBS. The following processes were carried out as described by Ferreira, C. [7].

E. ATP Level

The intracellular ATP level was measured with BacTiter-Glo™ Microbial Cell Viability Assay. Procedures for this assay have been described in detail elsewhere[8].

F. Data Analysis

SPSS 20 software was used for statistical analysis. GraphPad prism 6 was used for graphing and processing.

III. RESULTS

A. Determination of the Bactericidal Concentration of PHMB and BCDMH in PB

Quantitative bactericidal analysis and nutrient agar culture counting methods were used to assess the effects of different disinfectants on *S. aureus*, and the results are shown in Table I. When the bacteria were treated with PHMB or BCDMH alone, the bactericidal activity met Technical Standard for Disinfection (China 2002) for a disinfectant (KL ≥ 5) at 320 mg/L and 60 mg/L, respectively. Therefore, these concentrations were used as disinfectant controls in the following experiments.

Next, PHMB and BCDMH were combined at different ratios (as PB). The killing activity of PB containing 10 mg/L BCDMH and 10 mg/L PHMB was the same as that of 320 mg/L PHMB and 60 mg/L BCDMH alone; thus, this PB composition was used for subsequent analyses. The effective dose of BCDMH in PB was lower than that in the BCDMH control, and the effective dose of PHMB in PB was much lower than that in PHMB control, suggesting a synergistic effect between PHMB and BCDMH.

TABLE 1. DETERMINATION OF THE BACTERICIDAL CONCENTRATIONS OF PHMB AND BCDMH IN COMBINATION (PB). (N=3)

PHMB (mg/L)	KL	BCDMH (mg/L)	KL	PB (mg/L)		KL
				PHMB	BCDMH	
20	3.09±0.12	15	0.24±0.03	10	10	5.01±0.09
40	3.92±0.03	30	0.46±0.07	8	10	4.72±0.15
80	4.75±0.04	45	1.65±0.08	15	10	5.52±0.23
160	4.87±0.08	60	6.41±0.10	10	8	4.83±0.16
320	5.60±0.11	75	7.07±0.04	10	12	5.08±0.27

B. Effects of Time, pH, Temperature and BSA on Bactericidal Activity

The effect of action time was determined by suspension quantitative sterilization method. The results are shown in Figure 1. Compared with BCDMH, the bactericidal effect of PB was higher than that of BCDMH in first 6min, and no significant difference after 9min. PHMB alone can quickly kill microorganisms and its KL is 0.56 higher than that of PB in 12 min, standing for significant differences. It was

noticeable that PHMB amount used here is 16 times that of PB (10 mg/L PHMB+ 10mg/L BCDMH).

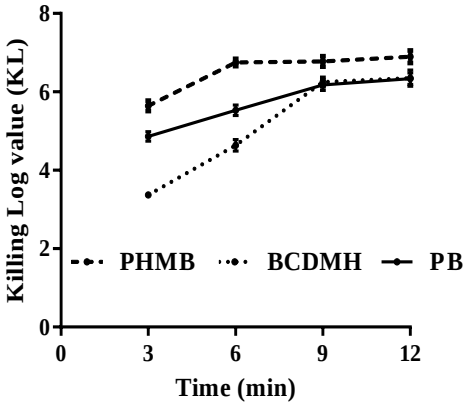

Figure.1. Effect of bactericidal time on killing of *S. aureus* (n=3, P<0.05)

On the basis of the above results, the mixture of 10 mg/L PHMB and 10mg/L BCDMH was determined as suitable concentration for next experiments. Quantitative bactericidal tests of suspension at different temperatures (0 ~ 40 ℃), quantitative bactericidal tests of suspension with different pH values (3.0 ~ 11.0) and quantitative bactericidal tests of suspension with different amount BSA were carried out separately. In all groups action time kept for 3min. The results are shown in Figure 2. As can be seen from Figure 2A, pH of the disinfectants had significant effects on the bactericidal activity. In pH 3.0 ~ 5.0 range, acidic environment, the bactericidal activity of PB was much better than alkaline or neutral environment. Best bactericidal activity occurred when pH was 5.0, and KL was up to 5.64. Figure 2B shows that temperature had no significant effect on bactericidal activity. Figure 2C shows that BSA can impede bactericidal activity and impediment increased as BSA concentration increased. When BSA in the suspension increased to 0.6%, the bactericidal effect was significantly decreased, but no significant difference existed between 0.6% group and 0.9% group.

C. SEM Analysis

Scanning electron micrographs of *S. aureus* treated with PB was shown in Figure 3. Figure 3 showed that the surface of untreated *S. aureus* was relatively smooth and had less protruded; treated *S. aureus* had more granular protrusions on the surface.

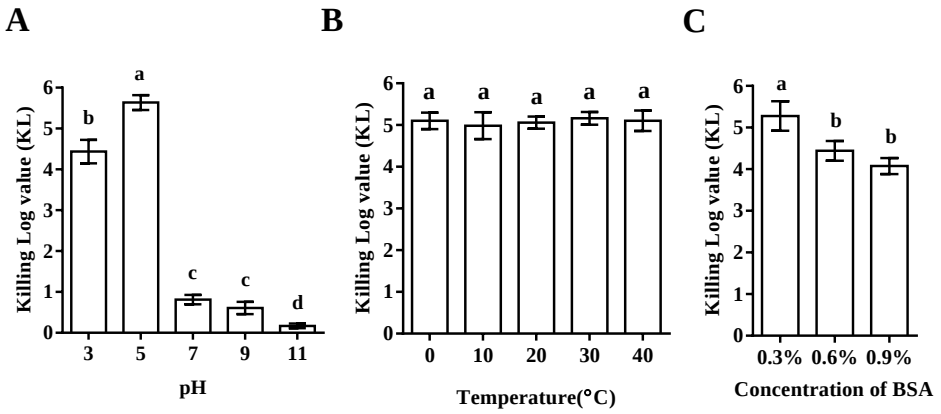

Figure 2. Effects of pH, temperature and BSA on bactericidal activity (n=3, P<0.05)

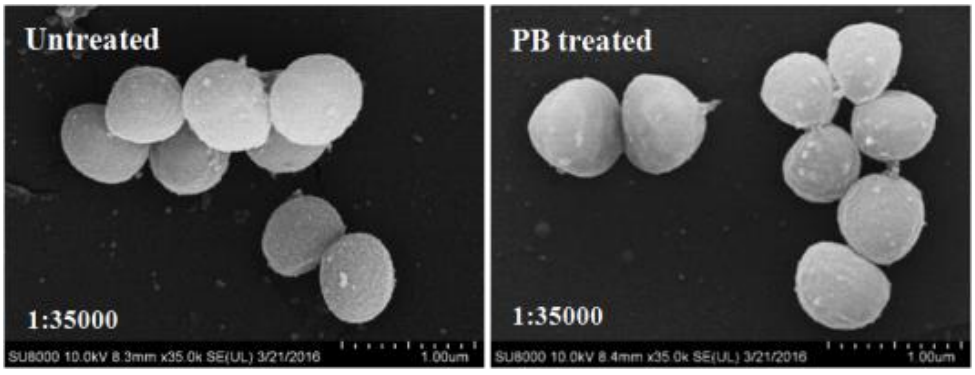

Figure 3. Scanning electron micrographs of *S. aureus* cells

D. Quantitation of Intracellular ATP

We measured intracellular ATP levels before and after PB treatment using BacTiter-Glo Microbial Cell Viability Assay. ATP levels were quantified and normalized to that of untreated cells. Figure 4 shows that intracellular ATP levels in all three treatment groups exhibited rapid decline at similar kinetic rates. The rate of decrease in ATP level in BCDMH group was about 6 times faster than that in PHMB group and 4 times faster than that in PB group within 5 min ~ 10 min after disinfectants treatment. During the period from 10 min to 20 min, decrease rate of BCDMH group slowed down. The decrease pattern of ATP level in PB group was similar to that in PHMB group.

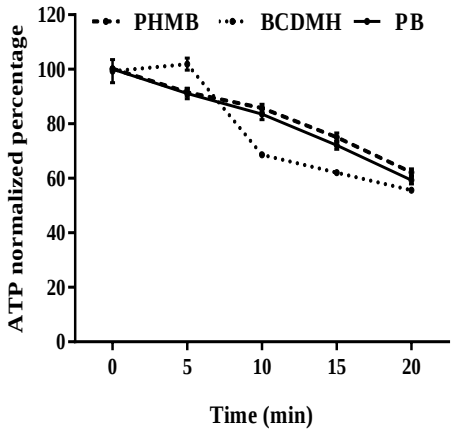

Figure 4. Variety in intracellular ATP level caused by disinfectants

IV. DISCUSSION

Existing disinfectants have different drawbacks to varying severity. Bisbiguanides are not effective against some gram-negative bacteria, particularly Pseudomonadaceae and *Providencia* spp. [9, 10]. Chlorination is one of the oldest and most common disinfection methods for water treatment. However, this approach has some potential side effects, such as the formation of carcinogens and trihalomethanes [11]. Researchers hope that combinations of chemicals or physical agents may have physical or chemical synergistic effects, such that the toxicity and amount of residual disinfectant can be reduced [12, 13]. Gilbert [14] reported that the effect of quaternary ammonium compounds on *Pseudomonadaceae* could be improved by combining them with a chelating agent such as EDTA. Both PHMB and BCDMH possess strong bactericidal activity. PHMB has a good safety profile but is expensive, whereas BCDMH is inexpensive but is not safe, as it can cause contact dermatitis. Binary mixtures of these chemicals may show physical or chemical synergy. These synergistic effects will potentially decrease the necessary dose and reduce the pungent smell of BCDMH. Compared to BCDMH or PHMB alone, PB achieved a similar KL at much lower concentrations (10 mg/L PHMB and 10 mg/L BCDMH). The effective doses of PHMB and BCDMH in PB were 1/32 of PHMB alone and 1/6 of BCDMH alone, respectively, which indicates that PHMB and BCDMH have synergistic effects.

The pH value of the PB compound solution was 4.23 for itself, and PB compound can gave satisfied bactericidal effect in pH range from 4.23 to 5.0, suggesting that the optimum pH value of PB was 4.23 ~ 5.0. The temperature had no significant effect on the killing of *S. aureus*, indicating PB can be used in wide temperature range and is less affected by season. As other disinfectants, effect of PB is apt to be impaired by BSA，here as organic interferent, indicating that organic materials in environment will lower bactericidal activity of PB.

Broxton et al. reported that PHMB can rapidly bind to lipopolysaccharide and peptidoglycan of the cell wall, and in doing so, it disrupts the otherwise stabilizing presence of Ca^{2+}[14-16]. More granular protrusions on the surface of treated *S. aureus*, indicating that PB can result in some changes in surface structure, maybe, mainly by PHMB component in PB. This phenomenon is consistent with data from PB-treated *E. coli* obtained in our laboratory (unpublished data)

BCDMH is related to level of bacterial metabolism, and in turn, to the ATP level. BCDMH could lead the ATP level to decline rapidly in 5 ~ 10min. It is possible that Cell membrane destruction by PHMB in PB may promote this process.

V. CONCLUSION

In summary, PB shows some obvious advantage over both BCDMH and PHMB alone. It can reach similar bactericidal activity in relatively smaller amount, and have less environmental side-effects.

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