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*Original article*

# The study on bactericidal effect and ultrastructural alterations of chlorocresol nanoemulsion disinfectant against *Staphylococcus aureus*

**Y.F. Zhang[#], Y.W. Sun[#], X.H. Liu, Z.X. An, X.F. Yang**

College of Animal Science and Veterinary Medicine,
Henan Institute of Science and Technology, Hualan Road No. 90, Xinxiang City, Henan Province, 453003, China

## Abstract

Chlorocresol nanoemulsion disinfectant (CND) is an environmental disinfectant prepared with nanoemulsion as its drug carrier. This study aimed to investigate the bactericidal effect of CND on *Staphylococcus aureus* (*S. aureus*) and its effect on bacterial ultrastructure. The neutralizing effect of CND against *S. aureus* was first screened by suspension quantitative evaluation experiment procedure of neutralizer. Disinfection performance was evaluated by the determination of Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), quantitative bactericidal experiment, and comparative experiment of disinfection performance between 0.1% CND and 0.1% chlorocresol aqueous solution. Meanwhile, the effect of CND on the ultrastructure of *S. aureus* was investigated with scanning electron microscope (SEM) and transmission electron microscope (TEM) to preliminarily explore the bactericidal mechanism. The results showed that 3% Tween-80 in PBS could be screened as the neutralizer of CND against *S. aureus*. MIC and MBC were 100 μg/mL and 200 μg/mL, respectively. The bactericidal rates were all 100% when 0.06% and 0.08% disinfectant acted for 15 and 5 min, respectively. Furthermore, compared with 0.1% chlorocresol aqueous solution, the bactericidal effect of 0.1% CND was significantly enhanced ($p<0.01$). After treatment with CND for 10 min, SEM observation showed that the morphology of *S. aureus* cells were changed and the integrity destroyed. TEM observation showed that the cell shape changed, and the structures of the cell wall, cell membrane and cytoplasm were damaged in varying degrees. CND showed the strong bactericidal effect on *S. aureus* and could cause ultrastructure alterations of *S. aureus*.

**Key words:** chlorocresol, nanoemulsion, disinfectant, Staphylococcus aureus, bactericidal effect, ultrastructure

Correspondence to: X.F. Yang, e-mail: yangxuefeng2003@126.com., tel.: 86-18238637551, fax: 86-0373-3040718

[#] These authors contributed equally to this work.

## Introduction

Epidemic disease is an important problem that modern animal husbandry must face. The infection of pathogenic bacteria will cause death of livestock and poultry, reduce production performance and cause serious economic losses. Furthermore, some zoonotic pathogens and highly infectious pathogenic bacteria will also endanger public safety. Especially in recent years, various animal diseases emerge in endlessly and the transmission speed of diseases is also accelerating with the continuous improvement of modern animal husbandry intensification and the continuous increase of animal feeding density. These situations lead to increased morbidity and mortality in livestock and poultry. How to effectively and fully prevent and control the occurrence and spread of epidemic diseases has become an important problem to be urgently solved in the development of animal husbandry.

Many data show that disinfection is an important method to prevent the spread of infectious diseases and limits infection routes (Dancer et al. 2014, Han et al. 2015, Boyce 2016, Matsubara et al. 2021). Chlorocresol, p-chloro-m-cresol (PCMC) (Roede et al. 2021), is a phenolic derivative disinfectant (Hidber et al. 2020, Roede et al. 2021). It can be mainly suitable for the disinfection of livestock and poultry pens, vehicles, utensils and the environment. However, its development and clinical application as a disinfectant product are restricted due to its shortcomings, such as slight solubility in water and the odor of phenol (Hu et al. 2010). Chlorocresol Nanoemulsion Disinfectant (CND) solution has been prepared (Yang et al. 2016) by taking advantage of nanoemulsion drug carriers (Badruddoza et al. 2018, Hashemnejad et al. 2019, Abdelmonem et al. 2019, Chen et al. 2020). It has the characteristics of good water solubility, weak phenol smell, simple production process, etc. One of our previous studies had reported that CND exhibited strong bactericidal activity against *E. coli.* (Yang et al. 2016), and another study showed that CND had a strong fungicidal effect and could cause ultrastructure alterations of *Candida albicans* (Yin et al. 2020). To the best our knowledge, few studies have shown the effect and mechanism of the disinfectant on *Staphylococcus aureus* (*S. aureus*) as an environmental disinfectant. The aim of this study was to investigate the bactericidal effect and the effect on bacterial ultrastructure of CND on *S. aureus.*

## Materials and Methods

### Bacterial strain

*Staphylococcus aureus* ATCC6538 was used as recommended by the "Technical Standard for Disinfection" of China (Ministry of Health of the People's Republic of China, 2008). The bacterial strain was kept and provided by the Animal Pharmacology Laboratory of Henan Institute of Science and Technology, Xinxiang, China.

### Preparation of CND

CND was prepared as previously described by Yang et al. (2016).

### Screened experiment of neutralizer

Complete neutralization of disinfectants was important for the accuracy of a biocidal assay (Eissa et al. 2012). In the present study, neutralizer selected experiment was performed according to suspension quantitative evaluation experiment procedures of neutralizer (Ministry of Health of the People's Republic of China, 2008). An optimal neutralizer was screened out through the experiment from three solutions: 3% (weight/volume, w/v, the same below) Tween-80 in PBS, 0.5% sodium thiosulfate in PBS and 1% Tween-80 + 1% lecithin + 0.5% sodium thiosulfate in PBS, respectively. The bacterial suspension of *S. aureus* was prepared by mixing with equal volumes of the bacterial suspension and 3.0% (w/v) bovine serum albumin and its final concentration ranging from $2.5\times10^3$ to $1.5\times10^4$ CFU (colony forming units) /mL was used. CND with the concentration of 0.20% (weight/weight, w/w, the same below) was used in all assays. Eight groups were set. Group 1 was composed of 0.4 mL disinfectant and 0.1 mL bacterial suspension. At the end of the interaction between the two, the volume of 4.5 mL of standard hard water was added into the test tube to reach the volume of 5 mL. Group 2 consisted of 0.4 mL disinfectant + 0.1 mL bacterial suspension and 4.5 mL neutralizer. After the reaction of disinfectant and bacterial suspension, neutralizer was added. The volume of this reaction system was also 5 mL. Group 3 was composed of 4.5 mL neutralizer and 0.1 mL bacterial suspension. After 0.4 mL of standard hard water was put into the test tube containing the neutralizer and mixed well, bacterial suspension was added. The volume was also 5 mL. Group 4 was formed from 0.4 mL disinfectant + 4.5 mL neutralizer and 0.1 mL bacterial suspension. After the reaction of disinfectant and neutralizer, bacterial suspension was added. Group 5 consisted of 4.5 mL diluent and 0.1 mL bacterial suspension. After 0.4 mL standard hard water was put into the test tube contain-

ing the diluent and mixed well, bacterial suspension was added. Group 6 was a culture medium group, group 7 was a diluent group and group 8 was a neutralizer group. The last three groups were used to observe whether the culture medium, diluent and neutralizer were contaminated, respectively. The selected neutralizer was considered to be optimal when deviation rate of the colony count among group 3, 4 and 5 was less than 15% and no bacterial growth occurred in group 6, 7 and 8. The experiment was carried out in triplicate.

### Bactericidal effect

### Determination of Minimal Inhibitory Concentration (MIC)

To evaluate antibacterial activity of CND, the MIC of CND against *S. aureus* was determined by nutritional broth dilution method (Ministry of Health of the People's Republic of China, 2008). The specific operation was as follows: The CND was diluted with distilled water into series test solutions of 10, 30, 50, 100, 200, 300, 400 and 500 μg/mL, respectively, and then 2.5 mL test solution was added into the test tubes with 2.5 mL nutrient broth culture medium. A volume of 0.1 mL of *S. aureus* suspension with a density of about $10^8$ CFU/mL was inoculated into the nutrient broth medium test tubes containing disinfectants of different concentrations, and they were taken as the test groups. Disinfectant action concentrations were 5, 15, 25, 50, 100, 150, 200 and 250 μg/mL, respectively. *S. aureus* was inoculated into the nutrient broth medium test tube without disinfectant in the same way, which was used for the positive control group. At the same time, another test tube containing only nutrient broth medium was taken as the negative control group. The liquid from the test group, positive and negative control groups were respectively cultured for 24 h, and the bacterial growth was recorded. The lowest disinfectant concentration with no bacterial growth in the test group was the MIC of CND to *S. aureus*. At the same time, 1 mL of test bacterial solution was taken from each test group with no bacteria growth observed by eyes, and inoculated into the agar medium without disinfectant for culture, respectively. The bacterial growth was observed after 24 hours. The minimum disinfectant concentration with no bacterial growth was the minimum bactericidal concentration (MBC) of CND against the bacteria. The test was repeated 5 times, and the modes were taken as MIC and MBC values (Yang et al. 2012).

### Quantitative bactericidal performance of bacterial suspension

According to "Technical Standard For disinfection (2008 edition)" (Ministry of Health of the People's Republic of China 2008), the quantitative bactericidal experiment of bacterial suspension of *S. aureus* was performed at 20°C±1°C. The concentrations of CND were 0.05%, 0.06%, 0.08%, and 0.10% (w/w), respectively.

### Comparison of bactericidal effect

A comparative experiment of disinfection performance between the same concentration (0.1%, w/w) of CND and chlorocresol aqueous solution was also conducted by using the same method as described above for the quantitative bactericidal experiment of bacterial suspension. All experiments were conducted in triplicate, data of Killing log (KL) on CND and chlorocresol aqueous solution were expressed as mean ± standard deviation. Statistical analysis was performed by Student's t-test in SPSS 10.0 (SPSS Inc., Chicago, Illinois, USA). Significant differences were accepted at $p<0.05$.

### Effect of CND on ultrastructure of S. aureus

### Sample pretreatment

Five milliliters of *S. aureus* suspension in the above suspension quantitative bactericidal test were added into 45 mL of 0.06% CND and mixed evenly. After 10 min of action, 450 mL of neutralizer was quickly added and neutralized for 10 min. Then the liquid mixture was centrifuged at 4°C 6 000 r/min for 3 min, and the supernatant was discarded. The lower layer of the bacterial sediment was left on standby after it was washed with PBS solution and centrifuged for 3 times. In the control group, PBS was used instead of CND but was otherwise treated the same. Samples from the test and control group were divided into two parts to conduct scanning electron microscopy and transmission electron microscopy, respectively.

### Preparation and observation of SEM samples

Each above bacterial sediment of the test and control group was suspended and fixed with 2.5% glutaraldehyde for 12 h at 4°C, centrifuged at 6000 r/min for 3 min, respectively. Then the sediment was dehydrated with 30%, 50%, 70%, 80%, 90% and 100% gradient alcohol for 8 min, respectively. After resuspension with 100% alcohol for 8 min, one drop of bacterial suspension was evenly smeared on the slide and dried naturally. Finally, the dried samples were sputtered with a layer of gold. The morphological changes of *S. aureus* before and after disinfectant were observed and photographed under the scanning electron microscope (SEM, Quanta 200, FEI Company, USA).

Table 1. Quantitative bactericidal effect of chlorocresol nanoemulsion disinfectant (CND).

| Groups | Log value of viable bacteria concentration after disinfecting for different time | | | Killing log (KL) on *Staphylococcus aureus* (*S. aureus*) ATCC6538 after disinfecting for different time | | |
|---|---|---|---|---|---|---|
| | 5 min | 10 min | 15 min | 5 min | 10 min | 15 min |
| 0.05% Disinfectant | 3.69 | 2.93 | 2.00 | 3.79 | 4.54 | 5.47 |
| 0.06% Disinfectant | 1.96 | 1.45 | No | 5.51 | 6.02 | ≥7.47 |
| 0.08% Disinfectant | No | No | No | ≥7.47 | ≥7.47 | ≥7.47 |
| 0.10% Disinfectant | No | No | No | ≥7.47 | ≥7.47 | ≥7.47 |

No: no viable bacteria were found.

Table 2. Comparison of bactericidal effect.

| Disinfectant | KL on *S. aureus* ATCC6538 after disinfecting for different time | | |
|---|---|---|---|
| | 5 min | 10 min | 15 min |
| 0.1% CND | ≥7.47±0.00** | ≥7.47±0.00** | ≥7.47±0.00** |
| 0.1% chlorocresol aqueous solution | 4.15±0.07 | 5.30±0.08 | 5.990±0.09 |

Log value of viable bacteria concentration was 7.47 in the positive control group, and no bacterial growth occurred in the negative control groups.
Data is presented as mean ± standard deviation.
** $p<0.01$ (highly significant) compared between CND and chlorocresol aqueous solution.

### Preparation and observation of TEM samples

Each above bacterial sediment of the test and control group was pre-fixed with 2.5% glutaraldehyde and fixed with 1% osmic acid, respectively. It was successively dehydrated in 50% ethanol for 20 min, 70% ethanol for overnight, 90% ethanol for 20 min, and 90% ethanol for overnight. Next, 90% acetone and bacterial suspension were mixed in a volume ratio of 1:1 for 20 min. Then, the mixture was subsequently dehydrated with 90% acetone for 20 min and embedded in resin to prepare ultrathin slices. Eventually, the sections were stained with uranyl acetate-lead citrate solution. Observation and photographs were made using a transmission electron microscope (TEM, H-7500, digital CCD camera system, Hitachi Company, Japan) .

## Results

### Neutralizing agent of CND

When three solutions of 3% Tween-80, 0.5% sodium thiosulfate and 1% Tween-80 + 1% lecithin + 0.5% sodium thiosulfate separately served as the neutralizer of CND, the deviation rates of colony count among groups 3, 4, and 5 were 5.01%, 14.41% and 11.40%, respectively. However, the deviation rate of colony counts was the smallest when 3% Tween-80 in PBS was used as the neutralizer. The results indicated that 3% Tween-80 in PBS was more appropriately selected as the neutralizer of CND.

### MIC of CND against S. aureus

When the liquid from each group was respectively cultured, the bacterial growth was observed by naked eyes in the positive control and CND 5~50 μg/mL groups but neither in the CND 100~250 μg/mL nor in the negative control groups. The findings showed that the MIC of CND to *S. aureus* was 100 μg/mL. As the liquid from CND 100~250 μg/mL groups was cultured, no bacteria grew in 200~250 μg/mL groups. The results indicated that the MBC of the disinfectant for *S. aureus* was 200 μg/mL.

### Quantitative bactericidal effect of CND

In the quantitative bactericidal experiment, log value of 7.47 was obtained in the positive control group, but no bacterial growth in the negative control group. As shown in Table 1, with the increasing of the disinfectant concentration and contact time, log value of viable bacteria concentration decreased, whereas the corresponding KL increased. KL had been more than 5 when 0.05% CND contacted for 15 min, indicating a qualified bactericidal efficacy. Furthermore, the bactericidal rates were all 100% when 0.06% and 0.08% disinfectant acted for 15 and 5 min, respectively.

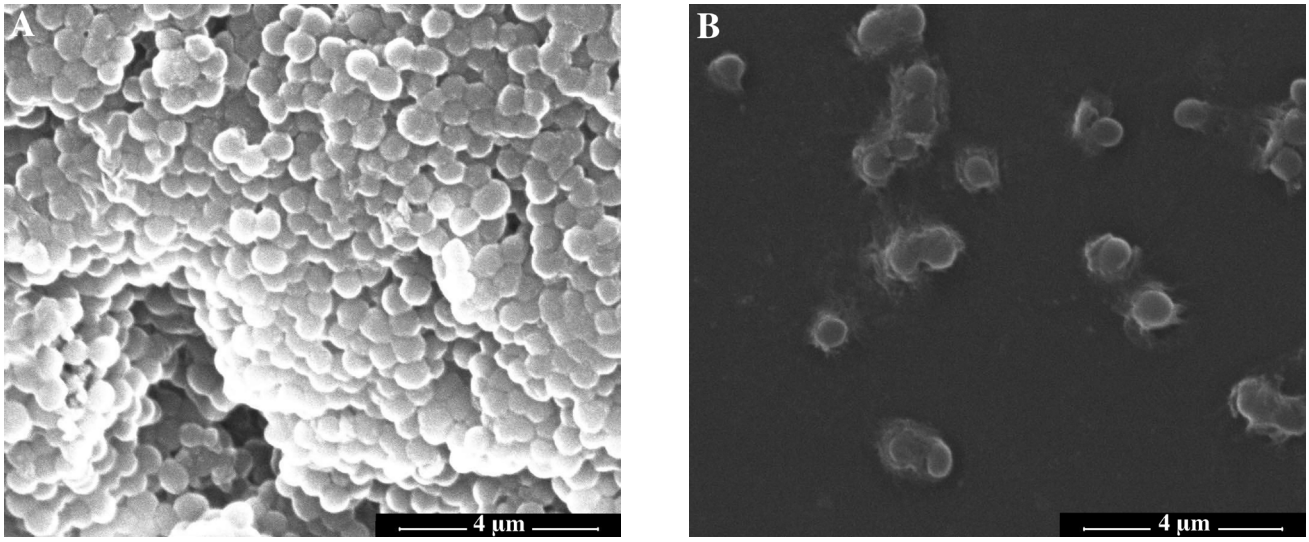


Fig. 1. Scanning electron microscope observation of the effect of CND on the ultrastructure of *S. aureus*.
A. *S. aureus* from the control group, ×12 000; B. *S. aureus* disinfected with CND for 10 min ×12 000

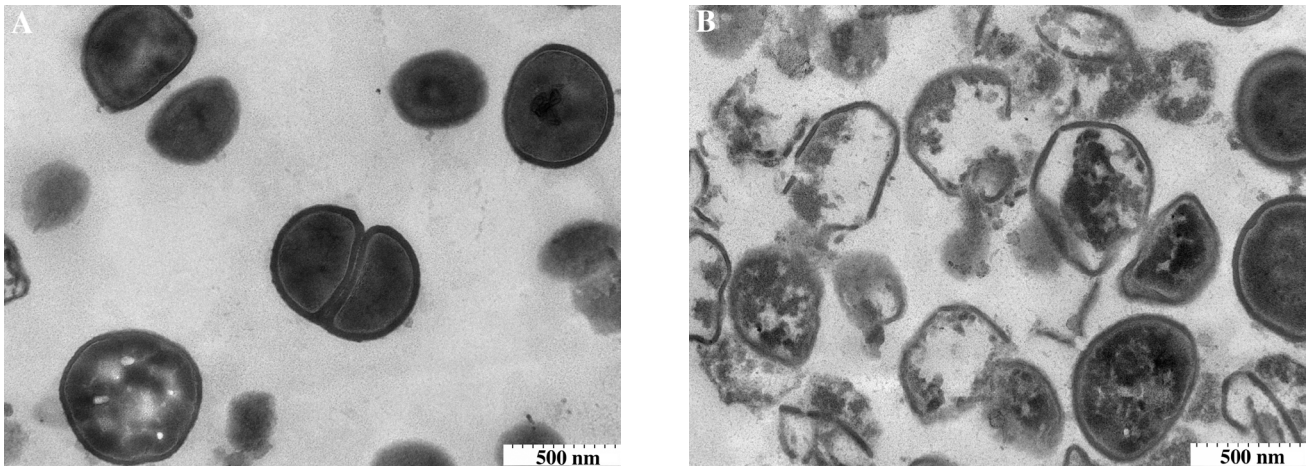


Fig. 2. Transmission electron microscope observation of the effect of CND on the ultrastructure of *S. aureus*.
A. *S. aureus* from the control group, ×12 000; B. *S. aureus* disinfected with CND for 10 min ×12 000

## Comparison of bactericidal effect between CND and chlorocresol aqueous solution

As shown in Table 2, KL of 0.1% CND were not only all more than 5, but also 100% bacteria were killed after disinfecting respectively for 5, 10 and 15 min. KL of 1.0% chlorocresol aqueous solution were more than 5 when the solution disinfected for 10 and 15 min, respectively. Furthermore, KL of the disinfectant after disinfecting for different time were all higher than that of the chlorocresol aqueous solution ($p<0.01$). The results revealed the bactericidal effect of the disinfectant was stronger than that of the chlorocresol aqueous solution.

## Effect of CND on cell ultrastructure of S. aureus

### SEM observation

The SEM observation results of *S. aureus* were shown in Fig.1. Normal *S. aureus* was round or spherical with smooth surface and complete cells (Fig. 1A). After the treatment with CND for 10 min, the surfaces of the cells were rough and had obvious structural changes. The cellular integrity was damaged since cellular content released around the cells, and the cells were broken and deformed (Fig. 1B).

### TEM observation

The TEM observation results of *S. aureus* were shown in Fig. 2. Normal *S. aureus* cell was spherical or approximately oval, the structure of the cell wall and cell membrane was complete, and the protoplasm

in different cells and the same cell was evenly distributed (Fig. 2A). After the treatment with CND for 10 min, TEM observation showed that the shape of *S. aureus* changed and the electron density of the cell wall increased. The protoplasm agglutination in varying degrees occurred in some cells, the integrity of the cell wall of some bacteria was destroyed, and most cells were completely broken (Fig. 2B).

## Discussion

The thorough and standardized disinfection of livestock and poultry houses and the environment is an important preventive measure to cut off the transmission route and source of epidemic diseases and kill pathogens. It is also the most effective and convenient method to prevent disasters before they happen. Our study aimed at investigating the bactericidal effect and the bacterial ultrastructure alterations caused by CND as a novel environmental disinfectant against *S. aureus*.

Some nanoemulsion disinfectants had been reported (Chepurnov et al. 2003, Wei et al. 2004, Ramalingam et al. 2013). Nanoemulsion ATB had been demonstrated to be an effective disinfectant for the Ebola virus (Chepurnov et al. 2003). Cetylpyridinium chloride-containing nanoemulsion disinfectant exposed to dental unit waterline biofilm for 1, 6, 12, 24, 48, and 72 h were observed high reduction of colonies and very low counts after 12 and 24 h (Ramalingam et al. 2013). Compound germicidal nanoemulsion disinfectants containing chlorhexidine, antimicrobial nanomaterial and other ingredients have been reported to have good bactericidal effect. The killing rates against *S. aureus*, *E. coli* and *C. albicans* on cloth strips disinfected respectively for 3, 3, and 5 min were all more than 99.90%. Moreover, the killing rates were all 100% for three microorganisms separately disinfected for 5, 5 and 10 min (Wei et al. 2004). In order to evaluate the disinfection effect of CND against *S. aureus*, the antibacterial activity and bactericidal effect were studied. The results showed that the MIC and MBC of CND against *S. aureus* were 100 μg/mL and 200 μg/mL, respectively. When 0.05% CND was applied to *S. aureus* for 15 minutes, KL was more than 5, and the disinfection effect met the requirements. Especially when 0.06% disinfectant acted on bacteria for 15 minutes, all bacteria were completely killed. These results indicated that CND had a strong killing effect on *S. aureus*. Furthermore, compared with 0.1% Chlorocresol aqueous solution, the bactericidal effect of 0.1% CND was significantly enhanced ($p<0.01$). The results suggested that nanoemulsified disinfectant could improve efficacy against microorganism more than unemulsified disinfectant, which was similar to the previous findings (Ramalingam et al. 2013, Horstmann et al. 2020). In this study, a new dosage form of CND was prepared with nanoemulsion as the drug carrier, and its bactericidal effect was stronger than the conventional dosage form of chlorocresol, which may be mainly caused by nanoemulsion as a special drug carrier. Chlorocresol was wrapped in the nanoemulsion and the nanoscale drug had small particle size. The reduced in the size molecules may easily penetrate the bacterial cell wall, thus increasing the intracellular drug concentration and antimicrobial activity (Horstmann et al. 2020). It was reported that the nanoemulsion structure itself had also extensive bactericidal activity (Hamouda et al. 1999, Hamouda et al. 2001). Additionally, our previous researches confirmed through on-the-spot disinfection test that CND had strong clinical disinfection efficacy (Yang et al. 2016, Mu et al. 2016). These results so far were very positive. This shows the potential of clinical application of the novel nanoemulsion disinfectant.

Different microorganisms have different sensitivity to the same disinfectant. Horstmann et al. (2020) reported that the nanoemulsion of chlorhexidine and commercial solution of chlorhexidine solutions were more effective on Gram-positive than Gram-negative bacteria ($p\leq0.05$). One of our previous studies showed that the disinfection was qualified when 0.08% CND acted on *E. coli* for 5 min, and 100% of the bacteria could be killed when 0.1% CND acted on the bacteria for 5 min (Yang et al. 2016). Our another study revealed that 0.8% CND for 10 min or 1.0% CND for 5 min killed 100% *Candida albicans* fungi (Yin et al. 2020). It could be seen that CND is more effective on the Gram-positive *S. aureus* bacteria than on the Gram-negative *E. coli* bacteria and *Candida albicans* fungi. Because of the widespread presence of various microorganisms in nature, the concentration of disinfectant was appropriately increased when CND was used for the comprehensive and thorough disinfection in the clinical practice.

In order to preliminarily explore the bactericidal mechanism of CND, the effect of CND on the ultrastructure of *S. aureus* was studied. When the concentration of 0.06% CND was applied to the bacterial suspension of S. aureus for 10 min in the above quantitative bactericidal experiment, some bacteria died, some of them were alive and some of their partial structural integrity was destroyed. Hence, the bacteria were treated with 0.06% CND to prepare microscopic slides and observe various ultrastructural changes of their cells. Under SEM, the cell morphology of *S. aureus* was changed and the integrity destroyed. Under TEM, the cell shape changed, and the structure of the cell

wall, cell membrane and cytoplasm were damaged in varying degrees. The results showed that CND could play a bactericidal role in destroying the integrity of the bacterial cell structure. The cell wall is a special structure of bacterial cells. Not only does it serve as the first defensive barrier of bacterial cells against external invasion, but also plays an important role in cell morphology maintenance, protection from external damage and the mediation of cell adhesion to host cells. Once the cell wall or membrane was damaged, the bacteria would lose inherent morphology, and their metabolism would be affected, resulting in damage or death. One of the bactericidal mechanisms of CND against *S. aureus* may be to alter the ultrastructure of the bacterial cells. However, other mechanisms of action need to be further investigated, encouraging the direction of future pharmacological studies.

## Conclusions

This study investigated the bactericidal efficacy of CND against *S. aureus* evaluated by MIC and MBC, the bactericidal action and disinfection performance. The effect of CND on ultrastructure of *S. aureus* was also studied with SEM and TEM. Our findings revealed that CND has a strong bactericidal effect on *S. aureus* and causes ultrastructure alterations of the bacterial cells. It can be considered as a promising candidate for a powerful environmental disinfectant.

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