Effect of New Dayuan powder on methicillin-resistant Staphylococcus aureus biofilms \textit{in vitro}

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\begin{abstract}
\textbf{OBJECTIVE:} To observe the effects of New Dayuan powder (NDYP) on Methicillin-resistant Staphylococcus aureus (MRSA) biofilms and the embedded bacteria \textit{in vitro}.

\textbf{METHODS:} 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assays were used to study the effects of NDYP on developing MRSA biofilms: 100 $\mu$L of bacterial culture and 100 $\mu$L drug solution were added to wells of 96-well plates. After 24 h of incubation, the plates were washed and XTT-phenazine methyl sulfate (PMS) was added to enable counting of the number of live bacteria in biofilms using a microplate reader. XTT assays were also used to explore the effects of NDYP on mature MRSA biofilms: 100 $\mu$L of bacterial culture were added to wells of 96-well plates. Bacteria were cultured in the plates for 24 h, and then drug solution was added. The plates were cultured for another 24 h, and then XTT-PMS was added to detect the number of live bacteria in the biofilms. Scanning electron microscopy (SEM) was used to observe the effects of NDYP on mature MRSA biofilms: washed and sterilized glass coverslips were added to 24-well plates. Bacterial culture was added. After 24 h of incubation, drug solution was added. After another 24 h of incubation, the samples were observed by SEM.

\textbf{RESULTS:} XTT assays showed that the number of live bacteria in both developing and mature MRSA biofilms decreased significantly ($P < 0.01$) after the administration of NDYP. SEM images showed that NDYP could destroy the structure of the bacteria and resulted in uneven thickness of MRSA biofilms.

\textbf{CONCLUSION:} In vitro, NDYP has obvious inhibitory effects on the formation of MRSA biofilms and on mature biofilms.
\end{abstract}

\textit{INTRODUCTION}

Antibiotic resistance has become a major public health issue worldwide. The appearance of multidrug resistant bacteria threatens everyone. Methicillin-resistant Staphylococcus aureus (MRSA) is a common bacterium in the clinic. Statistics from China Antimicrobial Surveillance Network CHINET in 2017 showed the prevalence of methicillin-resistant strains was 35.3\% in Staphylococcus aureus on average, nationwide.\(^1\) MRSA
shows resistance to the whole group of β-lactam antibiotics. In recent years, it was reported that many MRSA strains have acquired resistance to multiple antibiotics. Biofilms are a group of microorganisms where cells stick to each other on a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substances. Biofilms can protect MRSA from antibiotics and host immune defenses. Thus, they play an important role in prolonging the duration of infection and promoting colonization. Biofilms have become a big challenge to physicians and have shown clinical recalcitrance to antimicrobial treatment. Dayuan Yin was developed by Wu Youke to treat the syndrome of pathogen hidden in the pleuropneumonic interspace, which was similar to the manifestations of patients infected by antibiotic-resistant bacteria through clinical observations. New Dayuan powder (NDYP) is modified from Dayuan Yin and it has been used in our department as an experiential prescription for the treatment of antibiotic-resistant bacterial infection. The results have been very satisfactory. Clinical research has reported that the combined application of NDYP and antibiotics could enhance the clearance rate of antibiotic-resistant bacteria in patients with pulmonary infections, and increased the success rate at which patients could be removed from a ventilator, but the mechanism remains unknown. In this study, we aimed to explore the effects of NDYP on MRSA biofilms in vitro.

MATERIALS AND METHODS

Materials
NDYP is a traditional Chinese medicinal formula produced in granular form by Sichuan Neo-Green Pharmaceutical Technology Development Co., Ltd. (Chengdu, China), with good manufacturing practice standards. The granules contain extracts of 10 Traditional Chinese Medicines (indigo naturalis, Fructus Tsaooko, etc.) and no other ingredients are added.

LB medium contained 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride dissolved in ultrapure water with the pH adjusted to 7.2-7.4. LB medium was supplemented with 2.5 g/L of glucose to facilitate the growth of MRSA biofilms. Vancomycin (VI-ANEX S.A.) was prepared in sugar-supplemented LB medium to make a stock solution of 10 μg/mL and was used at dilution range 10-0.31 μg/mL. 2, 3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) powder was purchased from Sigma (St. Louis, MO, USA). It was added to sugar-supplemented LB medium to prepare a 1 mg/mL solution and was fully dissolved by heating in a water bath at 70 °C. The solution was prepared just before use, and exposure to light was avoided. Phenazine methyl sulfate (PMS) powder was purchased from Sigma (St. Louis, MO, USA). It was dissolved in ultrapure water to prepare a 3.06 mg/mL solution, which was subsequently filtered and sterilized using a 0.22-μm microporous membrane and stored in a refrigerator at 4 °C in the dark. Phosphate-buffered saline (PBS) was used to remove suspended bacteria from plates.

MRSA strain ATCC 43300 was used as the test bacterial strain and was purchased from the American Type Culture Collection (Manassas, VA, USA). A microplate reader was purchased from Bio-Rad (Hercules, CA, USA). It was used to determine OD values to evaluate the number of live bacteria present in biofilms. Scanning electron microscopy (SEM) was purchased from Hitachi (Tokyo, Japan). It was used to observe the state of bacteria in biofilms.

Preparation of drug solutions and bacterial cultures

Preparation of NDYP alcohol extract: NDYP granules were accurately weighed and dissolved in ethyl acetate. The solution was centrifuged at 166 × g 1000 r/min for 5 min, after which the supernatant was transferred to a beaker. When the collected ethyl acetate supernatant was visually clear and colorless, it was evaporated to powder using a rotary evaporator and the solid was dissolved in absolute ethanol to prepare a concentrated solution with a granule concentration of 5 g/mL. The solution was stored at 4 °C. Sugar-supplemented LB medium was used to prepare six concentrations of NDYP extract (25, 50, 100, 150, 200 and 250 mg/mL) for experimental use.

Preparation of NDYP aqueous extract: the remaining drugs from the ethyl acetate extract preparation were dissolved in water, and the aqueous solution was centrifuged to remove impurities. Next, the supernatant was evaporated in a water bath and then the resulting solid was dissolved in LB medium to prepare a 250 mg/mL solution. The solution was filtered and sterilized using a 0.22-μm microporous membrane and was then aliquoted into Eppendorf tubes and stored at 4 °C. Sugar-supplemented LB medium was used to prepare six concentrations of the extract (12.5, 25, 50, 75, 100, and 125 mg/mL) for experimental use. The osmotic pressure of the drug solution was in accordance with the requirements for bacterial growth. 125 and 250 mg/mL are respectively the maximum concentrations of aqueous and ethanolic extracts we can prepare in the experiments.

Preparation of bacterial culture: a single MRSA colony was picked from a plate and inoculated into 3 mL of LB medium. The culture was grown at 37 °C with shaking at 280 r/min for 16 h. The absorbance of the culture at 600 nm (OD600) was measured and it was subsequently diluted with LB medium containing 0.25% glucose to OD600 = 0.1 for later use.

Detection of the number of live bacteria during the formation of MRSA biofilms

One hundred microliters of diluted bacterial culture
petted off and discarded. Then, the glass with attached tants in each well of the 24-well plate. Next, 40 μL of medium was added to each well in columns 1 and 12 and rows 1 and 8. After incubating at 37 °C for 24 h at constant humidity, the plate was washed twice with 0.9% sodium chloride to remove suspended bacteria from the wells. Next, 40 μL XTT-PMS was added to detect the number of live bacteria in biofilms, with the OD values at 450 and 655 nm measured using a microplate reader after 1-2 h.

Detection of the number of live bacteria in mature MRSA biofilms

One hundred microliters of diluted bacterial culture (OD600 = 0.1) were added to each well in columns 2-11 of two 96-well plates which was purchased from Corning Incorporated (Corning, NY, USA), and 200 μL of medium was added to each well in columns 1 and 12 and rows 1 and 8. Bacteria were cultured in the plate at 37 °C for 24 h at constant humidity to form mature biofilms. Next, 100 μL of the drug solutions were added at the same concentrations as described above, with the blank control group also treated in the same as described before. The plate was cultured at 37 °C for another 24 h at constant humidity. After washing the plate, XTT-PMS was used to stain the biofilms, and OD values were measured using a microplate reader, as described above.

Observation of bacteria in mature biofilms by SEM

To inspect the impact of NDYP on mature MRSA biofilms, we observed the biofilms by SEM. First, a washed and sterilized piece of glass (0.18 cm × 0.18 cm) was added to each well of a 24-well plate. Next, 1 mL of bacterial culture was added to each well, and a biofilm was cultured according to the method described above to allow mature biofilms to grow on the glass pieces. A blank control group and a drug group were set up, with 1 mL of drug solution added in the drug group. The plate was cultured in an incubator at 37 °C under constant humidity for 24 h. Subsequently, the supernatants in each well of the 24-well plate were carefully pipetted off and discarded. Then, the glass with attached biofilms was carefully rinsed three times with PBS to remove suspended bacteria. After pre-fixation, post-fixation, dehydration, tert-butanol replacement, lyophilization and gold spraying, the biofilms were observed by SEM and photographed.

Statistical analysis

The experimental data were analyzed using SPSS 20.0 (SPSS Inc. Chicago, IL, USA). They are presented as the mean ± standard deviation (x ± i). One-way analysis of variance was used for data analysis. A pairwise comparison was performed by least significant difference if the variances were equal. Dunnett’s T3 test was used if the variances were unequal, and differences were considered significant at P < 0.01.

RESULTS

**XTT assays**

XTT assays were employed to investigate the effect of NDYP and vancomycin on the formation of MRSA biofilms. Live bacteria in the formation period of MRSA biofilms could be inhibited by vancomycin in the concentration range 5-10 μg/mL, aqueous extracts of NDYP in the concentration range 100-125 mg/mL, and ethanolic extracts in the range 100-250 mg/mL (Figure 1; Table 1). Thus, vancomycin was the most effective drug.

However, once the MRSA biofilms became mature, the effects of vancomycin on the inhibition of bacteria were reduced. On the contrary, both aqueous and ethanolic extracts of NDYP could still significantly decrease the number of live bacteria embedded in the mature biofilm matrix (Figure 2, Table 1).

**SEM**

The mature MRSA biofilms in the blank control group were thick. The bacteria embedded in them were tightly linked to each other, and filaments could be observed between them. They had morphological characteristics including orderly arrangement, smooth surface, normal shape, and uniform size. The features of MRSA biofilms in the vancomycin-treated group were similar to those in the blank control group. In the aqueous NYDP extracts-treated group, the distribution of the MRSA biofilms was uneven (Figures 3C and 3D respectively represent the thick and thin parts). Some
of them were covered by a membrane and the structures of some bacteria were destroyed. In the NDYP ethanolic extracts-treated group, the MRSA biofilms showed a loose and even structure (Figures 3E and 3F represent the even thickness), and it was found that some of the bacteria became larger than normal in the division phase. Meanwhile some of the bacteria were ruptured and shriveled. The above findings showed that both aqueous and ethanolic extracts of NDYP had inhibitory effects on mature MRSA biofilms and the latter was more effective; it could destroy the structure of biofilms and eliminate the bacteria embedded in them.

**DISCUSSION**

MRSA is a class of antibiotic-resistant bacteria, and biofilm formation is a means by which bacteria tolerate antibiotics. There are some differences between antibiotic resistance and tolerance. Resistance is an irreversible change to the genome of the bacteria, while tolerance is a reversible state of the bacteria that enables them to survive antibiotic treatment. A number of factors contribute to antimicrobial tolerance, such as slow growth rate, the failure of the drug to penetrate a biofilm, physiological changes, and gene expression or repression in the biofilm mode of growth. Traditional antibiotics
can efficiently kill bacterial growth and division stages, but are very inefficient in killing nonproliferative bacteria, which results in an inherent insensitivity of biofilms to antibiotics; indeed, resistance of biofilms to antibiotics is >1000-times that of suspended bacteria. Unlike suspended bacteria, bacteria in biofilms have many characteristics that make it difficult to eradicate them, for example, slow growth rate and the ability to resist host defenses and antimicrobial treatment. They are phenotypically distinct from their planktonic counterparts. Biofilm-embedded bacteria are in a slow-growth state or latency period. Therefore, antimicrobial treatment can suppress the symptoms of infection by killing suspended bacteria, but usually fails to eradicate bacterial cells embedded in a biofilm. Studies have shown that the development of many infections depends on the formation of a biofilm. The effect of biofilm formation on infection might have been underestimated, especially in chronic infection. The clinical significance of bacterial biofilms is particularly obvious in chronic infections, either for surface associated bacteria or suspended bacteria. Biofilm-related disease can lead to increased patient morbidity and mortality, and it also means a greater financial burden for healthcare. Biofilm-related infections also contribute to the generation and spread of antibiotic resistance in hospitals. Staphylococcus is the most common pathogen in biofilm-associated infections. Clinical therapy of MRSA infection mainly relies on the use of glycopeptides, especially vancomycin, which has been considered the gold standard for therapy of MRSA infections. Long-term use of high-dose antibiotic combinations is recommended in biofilm-related infections. Once the antibiotic treatment stops, the bacteria can return to a growth state and repopulate in a biofilm, leading to recurrence of the infection. However, vancomycin has strong nephrotoxicity and its use is therefore limited in patients with renal impairment. Therefore, we have developed the idea of using NDYP combined with antibiotics to treat biofilm-related infections.

NDYP is modified from three components: Dayuan Yin from *On Plague Diseases*, Shengjiang San from *Treatise on Differentiation and Treatment of Febrile and Plague Diseases*, and Xiaochaihu Tang from *Shang Han Lun*. From the point of view of Traditional Chinese Medicine (TCM), unlike the traditional treatment of bacterial infections, which focuses on clearing away heat and detoxification, NDYP puts more emphasis on the method of dispersing with clearing, giving pathogenic factors a way to come out. Patients with biofilm-related infections are always in poor physical condition and often long-term bedridden or immunosuppressed. So, the infection generally presents as a chronic process with recurrence as a prominent feature. In TCM, most patients with the characteristics mentioned above belong to complexity of deficiency and excess. It is improper to use heat-clearing and toxin-removing drugs alone; they will fail to eliminate pathogenic factors and damage vital Qi in vain. When pathogenic factors are relatively exuberant, strengthening vital Qi is more likely to lead to lingering of the pathogenic factors. Under such circumstances, it is wise to imitate Xiaochaihu Tang, the method of dispersing with clearing. In this way we can achieve the purpose of eliminating pathogenic factors without damaging vital Qi. From the point of view of categorization by analogy, bacteria embedded in biofilms are in dormant states, which is similar to latent pathogenic factors. Dayuan Yin is the classic prescription for treating syndromes of pathogen hidden in the pleurodiaphragmatic interspace. NDYP was developed based on this idea.

The difficulty in the treatment of biofilm-related infections lies in the method of penetrating the biofilm barrier. Our studies have shown that the tolerance of bacteria to antibiotics is reversible in vitro. Antibiotic susceptibility can be restored if bacteria are released from biofilms. From the results of our experiments, vancomycin in high concentrations had obvious effects on the formation of MRSA biofilms. However, the effects on mature MRSA biofilms were significantly reduced.
The effects of aqueous and ethanolic extracts of NDYP on mature MRSA biofilms are surprising. A new finding in this study is that NDYP could both damage biofilm structure and inhibit MRSA embedded in biofilms. This finding is also in accord with TCM theory. It is expected that better results can be achieved if the combination of NDYP and vancomycin is used in patients with MRSA biofilm infections. Although the results of this study have shown that NDYP can destroy biofilm structure and inhibit bacteria embedded in biofilms in vitro, it has some limitations. In vitro biofilm assays might not accurately represent in vivo biofilms. In particular, host factors have many important implications for biofilm formation in vivo, while they are less appreciable in current models in vitro. Treatment regimens that are able to completely clear all biofilms within a well-established chronic bacterial infection remain to be determined.

REFERENCES