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Original article

Synergy of ambroxol with vancomycin in elimination of catheter-related *Staphylococcus epidermidis* biofilm *in vitro* and *in vivo*

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ABSTRACT

Central venous catheters are widely used in neonatal intensive care units (NICUs) nowadays. The commonest cause of catheter-related bloodstream infections (CRBSIs) is coagulase-negative staphylococci (CoNS). Ambroxol, an active metabolite of bromhexine, exhibits antimicrobial activity against strains producing biofilm and enhances the bactericidal effect of some antibiotic by breaking the structure of biofilm. In this study, we aimed to determine the effect of ambroxol with vancomycin on the biofilm of *Staphylococcus epidermidis* (*S. epidermidis*) *in vitro* and *in vivo*. In the *in vitro* study, the biofilm of *S. epidermidis* was assessed by XTT reduction assay and analysed by confocal laser scanning microscopy (CLSM). In the *in vivo* study, a rabbit model of CRBSIs was created by intravenous intubation with a tube covered with *S. epidermidis* biofilm. The rabbits received one of the following four treatments by means of antibiotic lock therapy: normal heparin, ambroxol, vancomycin, or vancomycin plus ambroxol each for 3 days. The microstructure of the biofilm was assessed by scanning electron microscopy (SEM). The number of bacterial colonies in the organs (liver, heart, and kidney) and on the intravenous tubes was measured on agar plates. Pathological changes in the organs (liver, heart, and kidney) were observed with Hematoxylin-Eosin staining. The ambroxol exhibits significant efficacy to potentiate the bactericidal effect of vancomycin on *S. epidermidis* biofilm both *in vitro* and *in vivo*. The antibiotic lock therapy using a combination of ambroxol and vancomycin reveals a high ability to eradicate *S. epidermidis* biofilms *in vivo*. These results provide the basis of a useful anti-infection strategy for the treatment of CRBSIs.

1. Introduction

In the last decades, the improved survival rate of preterm infants, especially the very low birth weight (VLBW) infants, was attributed to the perinatal medicine advances. Increased survival rate of VLBW infants can be partly attributed to routine practice of central venous catheters (CVCs) placement [1]. The use of CVCs provides an intravenous route for administration of hyperosmolar fluids and medications. However, it also increases the risk of local and systemic infection, and there are negative relations between the number of infections and gestational age and birth weight [2]. A major complication of these devices implantation is catheter-related bloodstream infections (CRBSIs).

CRBSI are the most common nosocomial infection, with high morbidity and mortality rates, leading to long-term hospitalization with high costs [3]. It has been increasingly recognized that bacterial biofilm development on the surface of the device is a significant virulent factor in CRBSIs [4]. Coagulase-negative staphylococci (CoNS) is the most frequent pathogens cause of CRBSIs, among which *Staphylococcus epidermidis* (*S. epidermidis*) was the most common species (24.24%–58%) [5–7]. As a skin commensal,

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S. epidermidis can migrate from the skin along the surface of the device into the body, forming a highly organized bacterial community known as biofilm within 24 h [8], and can be more resistant to antibiotics compared with non-biofilm-producing (planktonic) bacteria [9]. Antibiotic lock therapy (ALT) combined with a conventional systemic antibiotic is effective treatment for long-term CRBSIs, particularly for CoNS infection [10]. One approach to overcome the intrinsic antimicrobial resistance of biofilm bacteria is to enhance the penetration of antibiotic agents through the biofilm matrix.

Ambroxol [2-amino-3, 5-dibromo-N-(trans-4-hy-droxycyclohexyl) benzylamine], an active metabolite of bromhexine, is used as an expectorant and a bronchodecretolytic in the treatment of bronchial asthma and chronic bronchitis, and it is also used in the treatment of pulmonary alveolar proteinosis and infant respiratory distress syndrome [11]. Furthermore, it exhibits antioxidant and anti-inflammatory properties as it could reduce the production of inflammatory cytokines from bronchoalveolar macrophages, monocytes and granulocytes [12]. Recently, it was observed that ambroxol can damage the structure of mucoid *Pseudomonas aeruginosa* biofilms *in vitro* and attenuate the lung damage caused by biofilm-associated infection *in vivo* [13,14]. Moreover, it enhanced the ability of ciprofloxacin to eradicate *P. aeruginosa* biofilms in a ventilator-associated pneumonia (VAP) rat model [15]. However, the role of ambroxol against *S. epidermidis* biofilm in catheter-related infections remains unknown. According to the above-mentioned studies, we hypothesized that the ambroxol can break the *S. epidermidis* biofilm and the bactericidal effect of vancomycin can be enhanced by co-administration of ambroxol.

In the present study, we aimed to demonstrate the synergy of ambroxol and vancomycin against *S. epidermidis* biofilm in an *in vitro* biofilm model and *in vivo* experimental CRBSIs biofilm model. This is the first study to explore the antimicrobial activity of ambroxol on *S. epidermidis* biofilm and the enhancement effect on the antibacterial ability of vancomycin.

2. Materials and methods

2.1. Bacteria, antimicrobial agents and animals

The bacteria strain, *S. epidermidis* ATCC 35984 (RP62A), used in this study, was kindly provided by Dr. Qian Gao, FuDan University in Shanghai. Bacteria was streaked out on tryptic soy agar (TSA) (Oxoid, Cambridge, UK) from frozen stocks and subsequently inoculated into 5 mL of tryptic soy broth (TSB) (Oxoid) and allowed to grow overnight at 37 °C with agitation (180rm) as described previously [16]. The vancomycin (Vancocin, Eli Lilly, Japan) and ambroxol (China Pharmaceutical Biological Products Analysis Institute, Beijing, China) were used in this study. Antibiotic lock solutions were prepared in sterilised heparin (Shanghai No.1 Biochemical and Pharmaceutical CO. LTD, Shanghai, China).

Sixteen New Zealand White Rabbits of either sex, weighing 2.2–2.5 kg, were purchased from the Chongqing Medical University Laboratory Animals Center (Chongqing, China) and used after a 1-week adaptation period in our laboratory. The rabbits were housed separately in individual cages and were exposed to a natural light–dark cycle and given free access to food and water. All experimental and animal care protocols were approved by the Animal Care and Use Committee, Chongqing Medical University.

2.2. In vitro studies

2.2.1. Biofilm formation and treatment protocol

Starting from overnight cultures on TSB agar plates, several colonies were re-suspended in TSB to reach an optical density equal to 0.5 McFarland standards (Cobas Inocheck; Roche Diagnostics GmBH, Mannheim, Germany). This suspension was diluted 100 times in TSB as the initial inoculum. Then, 100 μL of the bacterial suspension was applied to each well of a flat-bottomed 96-well plate. Plates were incubated statically for 24 h at 37 °C to allow biofilm formation. Then, preformed biofilms were treated for 24 h under four different conditions: non-treated, with ambroxol, with vancomycin and with vancomycin plus ambroxol. The concentration of ambroxol was 1.875 mg/mL, this concentration was selected because it can destroy the biofilm structure of *S. epidermidis* in preliminary experiment (data not shown). The concentration of vancomycin was 2 μg/mL, this concentration was chosen as it has been previously published reports confirmed was the MIC for RP62A [16].

2.2.2. Evaluation of the biofilms

After treated for 24 h, the supernatants were discarded and the biofilms were washed thrice with sterile phosphate-buffered saline (PBS). For XTT (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) reduction assay, the method was modified from described by Cerca et al. [17]. The XTT solution was prepared in TSB, and incubation of the biofilms with the staining solution lasted for 3 h at 37 °C in the dark. Absorbance of the supernatant was measured at 450 nm using a VICTOR3™ Multilabel Plate Reader.

2.2.3. Biofilm staining and confocal laser scanning microscopy

Biofilms were grown on a 10-mm diameter polystyrene membrane for 24 h and were treated as mentioned previously. Biofilms on the polystyrene membrane were rinsed thrice with 1 mL PBS and were stained with SYTO 9 and propidium iodide (PI) according to the instruction of the LIVE/DEAD *Bac*light kit (Invitrogen Molecular Probes, USA). Bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. After staining 30 min at room temperature in the dark, biofilms were rinsed with PBS to remove the unattached dyes and observed using a CLSM system (Radiance 2000; Bio-Rad, Hemel Hempstead, UK) consisting of a microscope (Nikon, Tokyo, Japan) and a krypton-argon mixed-gas laser source. Signals were recorded using the green (excitation 488 nm, emission 515/30 nm) and red (excitation 568 nm, emission 600/50 nm) channels.

2.2.4. Biofilm characterisation

Image Structure Analyzer (ISA) software developed by Beyenal et al. [18], was used to estimate structural parameters such as thickness, textual entropy (TE), areal porosity (AP) (defined as the ratio of void area to total area) and average diffusion distance (ADD) (defined as the average of the minimum distance from each cluster pixel to the nearest void pixel over all clusters) [19].

2.3. In vivo studies

2.3.1. Biofilm development

0.2 mL of bacterial inoculum (turbidity equivalent to that of a 0.5 McFarland standard and diluted 100 times) were inoculated into the disposable sterile plastic scalp acupuncture tube of 2.0 mm in diameter, which was cut into a length of 10 cm. The catheter was incubated at 37 °C for 24 h without disturbance. During that time, bacteria adhered to the inner surface of these inoculation tubes and proliferated. Biofilm formation was confirmed by SEM as described later.

2.3.2. Rabbit infection model and drug administration

Surgical placement of CVCs was modified from previously described methods [20]. Briefly, rabbits were anesthetized by