Combination effects of baicalin with linezolid against *Staphylococcus aureus* biofilm-related infections: in vivo animal model

Zhongye Du¹, Jiahui Han³#, Jing Luo¹, Guan Bi², Tangjuan Liu¹, Jinliang Kong¹*, Yiqiang Chen¹*

¹Department of Pulmonary and Critical Care Medicine, First Affiliated Hospital of Guangxi Medical University, Guangxi Medical University, Nanning, China; ²Department of Intensive Care Unit, Second Affiliated Hospital of Guangxi Medical University, Guangxi Medical University, Nanning, China; ³Basic Medical Sciences, School of Public Health, Hengyang Medical School, University of South China, Hengyang, China

#Equal contributors  
*Equal corresponding author

**Key words:**  
*Staphylococcus aureus*, baicalin, linezolid.

**SUMMARY**

*Staphylococcus aureus* is a gram-positive bacterium that can produce biofilm, and biofilm-associated infections are difficult to control. Biofilm prevents antibiotics from penetrating and killing the bacteria. Combined use of antimicrobials is a common strategy to treat *S. aureus* biofilm-related infections. In this in vivo study, the clinically isolated strain of *S. aureus* 17546 (t037) was selected to establish a biofilm-associated infection rat model, and baicalin and linezolid were used to treat the infection. CFU counting was used to determine the bacteria within the biofilm, the biofilm structure was viewed using scanning electron microscopy (SEM), histopathology was performed, and inflammatory factors were analyzed by ELISA. Baicalin was efficient in destroying the biofilm and exerted a synergistic bactericidal effect when combined with linezolid. Based on these findings, baicalin combined with linezolid may be efficacious in controlling *S. aureus* biofilm-related infections.

Received April 01, 2023  
Accepted July 25, 2023

**INTRODUCTION**

*Staphylococcus aureus* (*S. aureus*) biofilm-associated infections are very concerning due to their ability to resist treatments (Choi et al., 2015). Biofilm prevents antibiotics from penetrating and killing bacteria inside the biofilm membrane, making infection arduous to control (Chen et al., 2015; Li et al., 2014) and causing a great financial burden to patients. Presently, biofilm-associated infection is mainly treated with implantation materials (Cevizci et al., 2015; Qin H et al., 2014; Behlau et al., 2011), by combining antibiotics (Parra-Ruiz et al., 2012; Chai et al., 2016), and by using Chinese herbal medicines (Chen et al., 2015; Zhang et al., 2017; Wong et al., 2010; Wang et al., 2008). Linezolid (Linezolid et al., 2006), a synthetic oxazolidinone antibiotic, was approved by the US FDA in 2000 for treatment of gram-positive bacterial infections. Linezolid inhibits bacterial protein synthesis, has an efficient antibacterial property on methicillin-sensitive or resistant *Staphylococcus*, vancomycin-sensitive or resistant *Enterococcus*, penicillin-sensitive or resistant *Streptococcus pneumoniae*, and exerts antibacterial activity against *mycobacterium* (Wang et al., 2015; Zhang et al., 2017). But few studies have reported using the combination of baicalin and linezolid for *S. aureus* biofilm-related infections.

The present study aimed to analyze the combination of baicalin and linezolid treatment in an in vivo rat model of bacterial biofilm-related infections. Further, the study planned to determine whether baicalin can destroy *S. aureus* biofilm and exert a synergistic antibacterial effect with linezolid.

**METHODS AND MATERIALS**

**Bacterial Strain and Reagents**

For this in vivo study, *Staphylococcus aureus* 17546 (t037) was obtained from the First Affiliated Hospital of Guangxi Medical University. Baicalin (Sigma-Aldrich, Burlington, MA, USA) and linezolid powder for injection (Pfizer, Madison, WI, USA) were dis-
solved in dimethyl sulfoxide (DMSO; Amresco LLC, Solon, OH, USA).

**Rat model of biofilm-related infection**

Bacteria were grown in Luria-Bertani broth (LB), and the growth suspension was diluted to about 1×108 cfu/mL. A polystyrene disk (1×1 cm²) was placed on the Jet Biofil 12-well plate (Thermo Fisher Scientific, Eugene, OR, USA) containing 2 mL of bacterial suspension. Planktonic bacteria were removed using sterile saline, and the medium was refreshed daily for 3 days. Sixteen wistar female rats, weighing 180-220 g, were randomly divided into four groups, each group consisting of 4 rats. The rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate at a dosage of 0.002 mL/g. The polystyrene disk with biofilms was rinsed with sterile physiological saline and implanted at the dorsal midline. The antimicrobial agents were immediately injected after the implantation of the disk containing biofilm. There were the linezolid group (40 mg/kg/12 h); baicalin group (100 mg/kg/12 h); linezolid plus baicalin group; control group (DMSO/12h) (Cai Y et al., 2009; Murillo et al., 2009). The drugs were administered every 12 h for 24 hours. After 24 hours, the implanted disks were removed at 48 hours and 72 hours to determine the bacterial count. The biofilm CFU counts were calculated by seeding serial diluting of biofilm suspension from the disk on LB agar. The experiment was replicated three times. One disk was observed using scanning electron microscopy. The dorsal tissue surrounding the disk was removed for histopathological examination. Two microliters of blood were drawn from the aorta abdominalis of each rat to determine C-reactive protein (CRP), procalcitonin (PCT), and *Staphylococcus enterotoxin A* (SEA). Animal experiments were performed following the regulations for the care and use of laboratory animals and were approved by the local authorities.

**Scanning electron microscopic (SEM) evaluation**

Each biofilm disk was washed with sterile saline, fixed in 2.5% glutaraldehyde, and dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, and 100%). Then the disks were dried at room temperature, metalized with gold, and subsequently analyzed under a scanning electron microscope (TM-1000; Hitachi, Tokyo, Japan) at 30 kV.

**ELISA for determining CRP, PCT, and SEA**

Two mL of rat blood was centrifuged at 12,000 rpm for 15 min at 4°C, and the serum was separated and added to the 48-well plates according to the manufacturer’s (Sigma-Aldrich) instructions. The standard solution was diluted using the doubling dilution method. 10 μl of sample and 40 μl of diluent were mixed and incubated at 37°C for 30 min, then washed 5 times and dried at room temperature. 50 μl of conjugate reagent was added and incubated at 37°C for 30 min, then washed 5 times and dried at room temperature. After allowing 15 min for coloration, 10 μl of stop buffer was added to arrest the chemical reaction. A quantitative analysis was performed by measuring the OD at 450 nm wavelength using a microplate reader (Multiskan; Thermo Fisher Scientific).

**Statistical analysis**

There were three experimental replicates and two technical replicates to validate reproducibility. All values were presented as mean ± standard error. The graphs were constructed using version 5.0 GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) examined the differences between groups for statistical significance. P-values of less than 0.05 were considered statistically significant.

**RESULTS**

**Synergistic antibacterial effect of baicalin and linezolid**

After 24 h, 48 h, and 72 h of treatment, baicalin or linezolid alone could not reduce the bacteria within the biofilm, but baicalin plus linezolid could reduce the bacteria. Viable bacteria counts were significantly decreased (P<0.05) in the baicalin plus linezolid group, but in the baicalin or linezolid group, the bacterial counts were not statistically different compared to the control group (P>0.05) (Figure 1).
linezolid group, whereas the baicalin plus linezolid group showed a milder inflammation. The treatment with baicalin plus linezolid markedly relieved the inflammation in the dorsal tissue. The dorsal tissue surrounding the disk was dissected for H&E staining. Many neutrophilic granulocytes were observed in the control group, baicalin group, or linezolid group under an optical microscope. But in the baicalin plus linezolid group, the neutrophilic granulocytes were obviously decreased, and many physalides were found, indicating that the inflammation was controlled and the tissues were restored (Figure 2).

**Scanning electron microscopy**

The images (Figure 3) after 24 hours, 48 hours, and 72 hours of treatment with baicalin or linezolid were viewed. The structure of the biofilm in the control, baicalin, and linezolid groups was denser than the baicalin plus linezolid group. More extracellular matrix and bacteria were observed on the disks. But the structure of the biofilm formed in the baicalin plus linezolid group was loose and contained fewer bacteria and less extracellular matrix than the control, baicalin, and linezolid groups, indicating that the administration of the combination of baicalin and linezolid exerted good bactericidal effect.

**Elisa for CRP, PCT, and SEA**

The CRP, PCT, and SEA levels were evaluated to examine the effect of the drugs on the inflammation of the biofilm-infected rats. Changes in CRP and PCT levels followed similar trends to those seen for the
viable bacteria counts. The CRP level (Figure 4A) and PCT (Figure 4B) in the baicalin plus linezolid group obviously decreased (P<0.05). However, the baicalin or linezolid group was not significantly different from that of the control group (P>0.05). After 72 hours of treatment with baicalin or linezolid, the relative amount of serum SEA of the rats in the different treatment groups was determined, and the serum level of SEA (Figure 5) in the baicalin plus linezolid group obviously decreased (P<0.05).

**DISCUSSION**

*S. aureus* is a common pathogen that causes biofilm-related infections (Costerton *et al*., 1987; Myles *et al*., 2012). Once embedded in a biofilm, the bacteria are wrapped in the extracellular matrix; then resistance is enhanced, avoiding the host immune clearance and the bactericidal effect of antibiotics (Parvizi *et al*., 2015; Tascini *et al*., 2023). Therefore, *S. aureus* biofilm-related infection has raised concern in recent years. For managing bacterial biofilm-related infections, the strategies adopted are as follows. First, biomedical materials should be improved. *S. aureus* colonizes easily on PVC or polyethylene conduits; therefore, materials with a smooth surface (Valk *et al*., 1995; Ellis *et al*., 1996), such as Teflon or high elastic silicagel should be selected to reduce the adhesion of *S. aureus* and thus the incidence of biofilm-related infection (Du, Z *et al*., 2019). Second, the choice of antibiotics is considered. Glycopeptide antibiotics, such as vancomycin and teicoplanin, and oxazolidinones, such as linezolid, have remarkable bactericidal effects on *S. aureus*. Third, Chinese herbal medicine is viewed. Recently, Chinese scholars conducted experimental studies for the treatment of biofilm-associated infections using traditional Chinese medicine (Feng *et al*., 2000). Banerjee *et al*. found that the Chinese herb *Andrographis paniculata* cleared the biofilm and thus associated infections (Banerjee *et al*., 2017). Additionally, studies reported that tea tree-tree oil exerted...
a significant bactericidal effect on S. aureus biofilm (Park et al., 2007). Combining Chinese herbal medicine and antibiotics in treating biofilm-associated infections is a promising therapeutic method. Scutellaria exerted antiseptic, anti-inflammatory, anticoagulant, and antithrombotic properties (Broncet al., 2007). Some in vitro experiments found that baicalin destroyed biofilms and showed a synergistic effect with levofloxacin in killing bacteria within biofilms (Luo et al., 2017). In this in vivo experiment, baicalin or linezolid alone did not reduce the number of bacteria within the biofilm. On morphological evaluation, the biofilm membrane structure in the control, baicalin, or linezolid group was intact, the surface of the extracellular matrix was thick, and the bacteria on the membrane adhered tightly. The surrounding tissue of the implanted disk was hyperemic and edematous, and exudates were found in that area. Microscopic observation revealed that the infiltration of granulocytes was dominant in the tissue. However, with the combinatory treatment of baicalin and linezolid, the colony counts decreased compared to the other groups. Under a scanning electron microscope, the structure of the biofilm was loose, and the extracellular matrix and the bacteria within the biofilm decreased significantly. The congestion and edema of the tissue surrounding the implants gradually vanished, and the exudate volume decreased. On histopathological examination, inflammatory cells decreased and vacuolated cells increased. These results indicated that baicalin effectively destroyed the S. aureus biofilm and worked synergistically with linezolid in killing bacteria within the biofilm to control the infection. The results of ELISA also confirmed that the combined therapy of baicalin and linezolid reduced the inflammatory reaction, and the serum inflammatory factors were decreased significantly. Baicalin is available at a low price and has low toxicity and few side effects. It is widely used in the treatment of upper respiratory tract infections, urinary tract infections, dysentery, hepatitis, and hypertension. In this in vivo experiment, it was further confirmed that baicalin was able to disrupt the S. aureus biofilm in vivo. But the mechanism of action in vivo is not yet clear. Some in vitro experiments confirmed that the effects of Chinese herbal monomers on the bacterial biofilm were achieved by inhibiting the QS system (Banerjee et al., 2017). Whether baicalin can exert an antibiofilm effect in vivo by inhibiting the QS system needs further confirmation. At the same time, the in vivo experiment involves pharmacokinetics. Our future in vivo experiments will focus on this pharmacokinetic aspect to provide more reliable support for clinical application.

Acknowledgments
This work was funded by the National Natural Science Foundation of China (Grant Number: 81060002, 81570006) and used the personal fund of Guangxi Zhuang from the Health Committee, Autonomous Region (Grant Number: Z20210909). The authors are grateful to Liang Yang, Ph. D (Nanyang Technological University, Singapore) and Hengzhuan Wang, Ph. D (Department of Clinical Microbiology, University Hospital of Copenhagen, Denmark) for their experimental advice and technical assistance throughout this study.

References
Baicalin and linezolid on biofilm-related infection


Zhao Q., Chen X.Y., Martin C. (2016). Scutellaria baicalensis, the golden herb from the garden of Chinese medicinal plants. Science bulletin. 61 (18), 1391-1398.