Adhesion and biofilm inhibiting properties of Chinese Herbal Formula SanHuang decoction against antibiotic resistant staphylococcal strains

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Research Article

Keywords: SanHuang decoction (SH), scanning electron microscopy, Staphylococcal

Posted Date: December 31st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-136408/v1>

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Version of Record: A version of this preprint was published at Scientific Reports on March 30th, 2021. See the published version at <https://doi.org/10.1038/s41598-021-86647-8>.

Abstract

Clinical study has shown that external soaking and washing with the Chinese herbal SanHuang decoction (SH) can control the orthopedic-biofilm related infections. However, the antibiofilm activities of SH *in vitro* have not been investigated. The aim of the current study was to explore the effect of SH on adhesion and biofilm formation of antibiotic-resistant *staphylococci* on titanium surface, and to explore its probable mechanistic effects on *staphylococcal* strains. Biofilm-forming ATCC 35984 (methicillin-resistant *Staphylococcus epidermidis*, MRSE) and ATCC 43300 (methicillin-resistant *Staphylococcus aureus*, MRSA) strains were used in this study. The minimum inhibitory concentrations (MICs) of SH and vancomycin against planktonic bacterial strains were determined by the broth microdilution method. Different sub-MIC of SH with TSB (Tryptic soy broth) were used as the basis for experimental grouping (SH group). TSB culture medium alone (TSB group) or TSB containing vancomycin (vancomycin group) incubated with bacteria were considered as the negative or positive control group, respectively. The inhibitory effect of different treatment on bacterial adhesion and biofilm formation were observed by the spread plate method, CV (crystal violet) staining, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). Real time PCR analysis was performed to determine the effect of SH on the expression levels of *ica AD* and *ica R* gene in ATCC 35984 during the biofilm formation. The strains were found to be susceptible to SH decoction with MIC and MBC values of 38.75 mg/ml and 77.5 mg/ml, respectively. The MIC values for vancomycin was 2.5 µg/ml. SH treatment with 1 MIC and 1/2 MIC could inhibit the bacteria adhesion on the titanium surface, showing only scattered bacterial adhesion from SEM. CLSM showed that SH with 1 MIC and 1/2 MIC could also inhibit the bacterial biofilm formation. The quantitative results of the spread plate method and CV staining showed that there was significant differences between the SH groups ($P < 0.05$). Further, with an increase in SH concentration, the inhibitory effect became more obvious at different culture time points, when compared with TSB control group ($P < 0.05$). Among the groups, vancomycin had the strongest inhibitory effect on bacterial adhesion and biofilm formation ($P < 0.01$). Meanwhile, with an increase in SH concentration, the expression levels of *ica A* and *ica D* decreased, and the expression of *ica R* increased correspondingly ($P < 0.05$). In conclusions, a certain concentration of SH can inhibit the adhesion and biofilm formation of antibiotic-resistant *Staphylococcal* strains on the titanium surface, but the effect was not as good as vancomycin. Its probable mechanistic activity may be through the inhibition of polysaccharide intercellular adhesin synthesis by down-regulating the expression of *ica AD* gene, thus inhibiting bacterial biofilm formation.

Introduction

Clinical study has shown that external soaking and washing with the Chinese herbal SanHuang decoction (SH) can control the orthopedic-biofilm related infections. However, the antibiofilm activities of SH *in vitro* have not been investigated. The aim of the current study was to explore the effect of SH on adhesion and biofilm formation of antibiotic-resistant *staphylococci* on titanium surface, and to explore its probable mechanistic effects on *staphylococcal* strains. Biofilm-forming ATCC 35984 (methicillin-resistant *Staphylococcus epidermidis*, MRSE) and ATCC 43300 (methicillin-resistant *Staphylococcus*

aureus, MRSA) strains were used in this study. The minimum inhibitory concentrations (MICs) of SH and vancomycin against planktonic bacterial strains were determined by the broth microdilution method. Different sub-MIC of SH with TSB (Tryptic soy broth) were used as the basis for experimental grouping (SH group). TSB culture medium alone (TSB group) or TSB containing vancomycin (vancomycin group) incubated with bacteria were considered as the negative or positive control group, respectively. The inhibitory effect of different treatment on bacterial adhesion and biofilm formation were observed by the spread plate method, CV (crystal violet) staining, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). Real time PCR analysis was performed to determine the effect of SH on the expression levels of *ica AD* and *ica R* gene in *ATCC 35984* during the biofilm formation. The strains were found to be susceptible to SH decoction with MIC and MBC values of 38.75 mg/ml and 77.5 mg/ml, respectively. The MIC values for vancomycin was 2.5 µg/ml. SH treatment with 1 MIC and 1/2 MIC could inhibit the bacteria adhesion on the titanium surface, showing only scattered bacterial adhesion from SEM. CLSM showed that SH with 1 MIC and 1/2 MIC could also inhibit the bacterial biofilm formation. The quantitative results of the spread plate method and CV staining showed that there was significant differences between the SH groups ($P < 0.05$). Further, with an increase in SH concentration, the inhibitory effect became more obvious at different culture time points, when compared with TSB control group ($P < 0.05$). Among the groups, vancomycin had the strongest inhibitory effect on bacterial adhesion and biofilm formation ($P < 0.01$). Meanwhile, with an increase in SH concentration, the expression levels of *ica A* and *ica D* decreased, and the expression of *ica R* increased correspondingly ($P < 0.05$). In conclusions, a certain concentration of SH can inhibit the adhesion and biofilm formation of antibiotic-resistant *Staphylococcal* strains on the titanium surface, but the effect was not as good as vancomycin. Its probable mechanistic activity may be through the inhibition of polysaccharide intercellular adhesin synthesis by down-regulating the expression of *ica AD* gene, thus inhibiting bacterial biofilm formation.

Postoperative infection is one of the serious complications impacting the clinical results of orthopedic surgery, and its inadequate management will lead to improper union of fracture, loss of limb function and even amputation, which will inevitably impose an economic burden to the patients' families and social medical insurance¹. According to the literature, the incidence of postoperative infections for closed fracture is about 1-2 %, but the incidence of an open fracture is significantly as high as 30 %^{1,2}. Studies have shown that 80% of clinical infections are related to bacterial biofilm formation (BBF), which plays an important role in the pathogenesis of refractory orthopedic infections³. Firstly, bacteria in a biofilm are very resistant to antibiotics, and their antibiotic resistance concentration is 1,000-fold higher than the MIC of the planktonic form, which has further attributed to the metabolic dormancy, altered microenvironments within the biofilm, and active resistance mechanisms^{3,4}. Secondly, the biofilm and its colonized pathogens can successfully defend the host's immune system, so that they cannot be phagocytized and cleared by polymorphonuclear leukocytes⁵. Therefore, inhibition of BBF is key to the successful treatment of orthopedic infections.

For refractory orthopedic infections related to BBF, in addition to thorough local debridement, another important option is a long-term application of systemic intravenous antibiotics⁶. In clinical practice,

however, BBF-related infections are often caused by resistant *Staphylococcus*, especially MRSE and MRSA, which makes the treatment of implant-associated infections with antibiotics still one of the key challenges for orthopedic surgeons^{2,4,6}. Hence, discovering alternative treatment methods for orthopedic infections linked to multi-antibiotic-resistant bacteria forming BBF is becoming more and more relevant.

For a long time, TCM (traditional Chinese medicine) has been widely used in the treatment of various infectious diseases in China, and many traditional or empirical prescriptions have been proved to have satisfactory clinical therapeutic effects⁷⁻¹⁰. The SanHuang decoction (abbreviated as SH) is one of the treatment methods widely used in our hospital for treatment of limb infections with bone and implant exposure. This decoction is composed of *Scutellaria baicalensis* Georgi, *Coptidis rhizoma*, *Cortex Phellodendri chinsis* and other Chinese herbs. According to the theory of traditional Chinese medicine, it has the heat-clearing and detoxing functions. Through external soaking and washing of the locally infected wound with SH, it was found that this decoction can effectively control the infections⁸⁻¹⁰. However, there has been no study to date that has explored the antibiofilm formation properties of the SH *in vitro*. Therefore, based on previously published clinical studies⁸⁻¹⁰, this study used MRSE and MRSA which are widely seen in orthopedic implant-associated infections, and investigated the effect of SH decoction on adhesion and biofilm formation of bacterial strains on titanium surfaces. Further, we also explored the probable mechanism behind its effect on biofilm formation.

Materials And Methods

Preparation of SH decoction. SH contains *Scutellaria baicalensis* Georgi (25 g), *Coptidis rhizoma* (25 g), *Cortex Phellodendri chinsis* (25 g), *Sophora flavescens* (20 g), *Lonicera Japonica* (20 g), *Forsythia suspensa* (20 g) and *Taraxacum mongolicum* Hand.-Mazz. (20 g) (Table 1). All the herbs were provided by the TCM pharmacy of Henan Luoyang Orthopedic-Traumatological Hospital following the requirement standards of the section one of Pharmacopoeia of the People's Republic of China (2015 edition). The procedure of preparing the SH aqueous extract is as follows: firstly, all herbs were tipped into a ceramic pot with 1000 mL pure water and soaked for 30 min. Secondly, the concoction was boiled at high heat for 30 min, and was maintained at a low flame until the aqueous extract reduced to 250 ml. Finally, aqueous extract was filtered with filter paper, and water extraction of the liquid was performed through Ø 22 µm filter. Based on the original herb dose, the initial liquid concentration of the extract solution was about 0.62 g/ml, which was stored in the refrigerator at -20 °C for the subsequent experiments.

Preparation of bacterial strains. In this study, the positive biofilm-forming strain ATCC 35984 (MRSE) and ATCC 43300 (MRSA) were used to evaluate the bacterial biofilm formation on titanium surface¹¹. The strains frozen in a -80°C refrigerator were rapidly thawed and inoculated on a tryptone soy agar (TSA) plate and cultured in an incubator at 37 °C for 24 h. A single colony was selected from the TSA and inoculated in a 50 ml centrifuge tube containing 10 ml TSB, the tube was incubated for 10 h at 37 °C with agitation at 100 rpm. Then, 25 µl of bacterial suspension was transferred to another sterile centrifuge tube containing 10 ml TSB and incubated at 37 °C for 14 h, after which 1 ml suspension was transferred

into a 1.5 ml centrifuge tube and the bacteria in the suspension was harvested by centrifugation for 5 min at 4°C and 1000 rpm (Sorvall TC6 centrifuge, Du Pont, Bad Nauheim, Germany). The precipitate was then washed three times with 0.15 M PBS (phosphate buffered saline) to remove the remaining TSB, and resuspended in sterile PBS to an optical density of 0.490 at 600 nm using Synergy HT multi-detection microplate spectrophotometer (Bio-tek, Winooski, VT), which corresponded to 10^9 CFUs (Colony forming units)/ml. Finally, the bacteria concentration was adjusted to 1×10^6 CFUs/ml by TSB, TSB-SH or TSB-Vancomycin.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination.

The broth microdilution method was used to determine the MIC and MBC of SH and vancomycin as recommended by the National Committee for Clinical Laboratory Standards Institute¹². An overnight culture of the strains were diluted 10-fold in TSB and incubated at 37°C until they reached exponential growth phase. Serial two-fold dilutions of SH (155 mg/ml to 4.84 mg/ml) or vancomycin (8 µg/ml to 0.0625 µg/ml) in TSB were prepared in a 96-wells plate with 190 µl per well. Ten microlitres of bacterial inocula of each strain containing 5×10^6 CFUs/ml was added in all wells. A number of wells with only TSB (without inoculum and drugs) in each plate were designed as blank control, and a number of wells with TSB and inoculum (without SH or vancomycin) were designed as negative control. The plates were incubated for 24 h at 37 °C. The MIC was detected following addition of 50 µl of INT (2-4-Iodophenyl-3-4-nitrophenyl-5-phenyl-2H-tetrazolium chloride) at a final concentration 0.2 mg/ml in all the wells and incubated for 30 min at 37 °C. Bacterial growth was determined by observing the color change of INT in the wells. Biologically active bacterial cells will reduce the colourless tetrazolium salt which act as an electron acceptor to a red-coloured formazan product¹³. Inhibition of bacterial growth is observed when the solution in the well remained clear after incubation with INT. MIC was defined as the lowest extract concentration that completely inhibits the growth of microorganisms. To determine MBC values, 10 µl of culture medium from the microtitre plate wells that showed no changes in color will be re-inoculated on agar plates. After 24 h incubation at 37°C, MBC was determined as the lowest concentration that yielded nil bacterial growth on agar plates. Each experiment was performed three times in duplicate.

Experimental grouping. Different sub-MICs of SH decoction were used as grouping basis. Only TSB incubated with bacteria were used as the negative control, and TSB containing vancomycin (2.5 µg/ml) and incubated with bacteria as the positive control. In total, our study had six experimental groups, namely the TSB group (bacteria cultured with only TSB), Vancomycin group (bacteria cultured with TSB containing vancomycin), 1/8MIC SH group (1MIC SH was diluted by 1/8 using TSB), 1/4MIC SH group, 1/2MIC SH group and 1MIC SH group.

Effect of different SH concentration on bacterial adhesion. Titanium discs of 10 mm in diameter and 1 mm in thickness were placed into a 48-well plate (Costar 3548, Corning, NY, USA), and six technical replicates were used for each group. *ATCC* 35984 and *ATCC* 43300 were diluted to a density of 10^6 CFUs/ml with TSB, TSB-vancomycin, or different sub-MICs of SH, and 1 ml of the cell suspension was added to the well containing the discs. The plates were incubated in aerobic conditions at a low shaking

rate of 100 rpm for 6 h. Then, the bacterial adhesion on the titanium surface were analyzed with the following methods.

The spread plate method: The discs were gently washed with PBS and transferred into a 10 ml glass tube containing 0.5 ml TSB. The tubes were then placed in an ultrasonic bath (B3500S-MT, Branson Ultrasonics Co., Shanghai, China) and the bacteria attached on the disc were dislodged by ultrasonication (5 min), then followed by rapid vortex mixing (Vortex Genie 2, Scientific Industries, Bohemia, NY, USA) at maximum power for 1 min. The vortexed solutions were serially diluted 10-fold and the final three dilutions were plated in triplicate onto TSA and then incubated at 37 °C for 24 h. Finally, the number of CFUs on the TSA were counted, and the number of bacteria in the biofilm was calculated and is expressed relative to the surface area of the sample (CFUs/mm²).

SEM assay: A sterile pipette was used to carefully remove the TSB from each well. The discs were transferred with forceps into another fresh 48-well plate and gently washed with PBS three times to remove loosely adherent bacteria. The discs were fixed in 2.5% glutaraldehyde for 2 h at 4 °C, then washed with PBS and dehydrated through a series of graded ethanol solutions (25, 50, 75, 95 and 100%). The samples were subsequently freeze-dried, sputter coated with gold, and observed using SEM (Joel JSM-6310LV, JEOL Ltd., Tokyo, Japan).

Effect of different SH concentration on biofilm formation. Titanium discs of 10 mm in diameter and 1 mm in thickness were placed into a 48-well plate, and six replicates were used for each group. *ATCC* 35984 and *ATCC* 43300 were diluted to a density of 10⁶ CFUs /ml with TSB, TSB-vancomycin, or different sub-MICs of SH, and 1 ml of the cell suspension was added to each well. The plates were then incubated in aerobic conditions at low shaking rate of 100 rpm for 24 h. Biofilm formation on the titanium surface were analyzed by the following methods.

CV staining assay: A sterile pipette was used to carefully remove the TSB from each well. The discs were transferred into another 48-well plate and gently washed three times with PBS. Then, 1 ml of 2.5% glutaraldehyde solution was added into each well for 10 min to allow fixation. The glutaraldehyde solution was removed and the wells were washed with PBS. Subsequently, PBS was removed and 2 ml of 0.1% (w/v) aqueous CV solution was added to each well followed by incubation for 20 min at room temperature. CV solution was discarded, and the wells were washed with PBS again and air-dried for 12 h in the dark. Next, the quantity of biofilm was analyzed by adding 30% acetic acid in a volume of 1 ml to each well to dissolve dye for 30 min, after which 200 µL of the dye solution were transferred into a 96-well microtiter plate, one group equaling one vertical row of the plate. The optical density at 492 nm was read using a plate reader (Synergy HT, BioTEK).

CLSM assay: After 24 h incubation, the TSB medium were removed carefully from each well, and the discs were transferred into another 48-well plate and gently washed with PBS three times. Then the wells containing the titanium discs were added with 500 µl combination dye (LIVE/DEAD BacLight bacteria viability kits, L13152; Molecular Probes, Eugene, OR, USA) in a dark environment at room temperature for

15 min and were subsequently analyzed with a CLSM (Leica TCS SP2; Leica Microsystems, Heidelberg, Germany). The live/dead kit containing two kinds of fluorescence dyes were used to distinguish the viable and non-viable cells under the fluorescence microscope, because dye SYTO 9 can make the viable bacteria with intact cell membranes display green fluorescence, whereas dye PI (propidium iodide) can make non-viable bacteria with damaged membranes display red fluorescence. The Leica confocal software was used to analyze the biofilm images. The images were acquired from random positions on the disc surface.

Effect of different incubation times on biofilm formation. The bacterial biofilm formation on the titanium surface was also observed at different incubation time-points. Titanium discs (10 mm in diameter and 1 mm in thickness) were placed into 48-well plates, 1 ml of the cell suspension with 10^6 CFUs/ml adjusted by TSB, TSB-vancomycin, or different sub-MICs of SH were added to each well and incubated at 37 °C for 6, 12, 18, 24, and 48 h. At the specified time-point, the quantitative analysis of bacterial biofilm formation on the titanium surface were observed by CV staining assay, as described above.

Real-time PCR analysis of *icaAD* and *icaR* transcription. The experiment had five groups, including the TSB, 1/8MIC SH, 1/4MIC SH, 1/2MIC SH and 1MIC SH group. *ATCC* 35984 was selected for the *icaAD* and *icaR* expression assays. Titanium discs (14 mm in diameter and 1 mm in thickness) were placed into 24-well plates, and 2 ml of the cell suspension with 10^6 CFUs/ml adjusted by TSB only or SH at different sub-MIC were added to each well and incubated at 37 °C for 24 h. Then, the bacterial biofilms on the disc surface were harvested together with the planktonic bacteria by sonication and transferred into the RNAProtect bacterial reagent (Qiagen, Germantown, MD, USA) to ensure RNA integrity. Bacteria were pelleted by centrifugation at 4 °C and 8000 g for 10 min. We further removed the protective reagent and resuspended the pellets in 0.8 mL TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 7.0) containing 100 g/ml lysostaphin (Sigma, St Louis, MO, USA). Total RNA was isolated using Axyprep Multisource Total RNA Miniprep Kit (Axygen bioscience, Union City, NJ, USA) according to the manufacturer's instructions. RNA was reversely transcribed to cDNA using the PrimeScript® RT reagent kit (TaKaRa, Shiga, Japan). Real-time PCR was performed on an ABI 7500 Fast machine according to the SYBR® Premix ExTM PCR kit (TaKaRa) kit. The reactions were performed using cDNA templates, and with specific forward and reverse primers. The amplification conditions were as follows: 50 °C for 20 s; 40 cycles of 95 °C for 15 s, and 60 °C for 1 min; then 95 °C for 10 min. The expression levels of *icaAD* and *icaR* were evaluated and normalized to the internal standard gene 16S rRNA. The quantification of gene expression was based on the CT value of each sample, which was calculated as the average of three replicate measurements for each sample analyzed. The primers used for the RT-PCR are shown in Table 2 ¹⁴.

Statistical Analysis. All the experiments were performed in triplicate. Data are expressed as mean $\pm$ standard deviation. The results were tested using a one-way analysis of variance with Tukey's multiple comparison tests. The differences observed between groups were considered to be significant at $P < 0.05$ using SPSS 19.0 software.

Results

MIC and MBC of SH decoction and vancomycin. SH was assessed on growth inhibitory ability against planktonic cells of standard strains. The potency was quantitatively assessed by the MIC and MBC values (Table 3). Among all the pathogen, *ATCC* 35984 and *ATCC* 43300 were found to be susceptible to SH decoction with MIC and MBC values of 38.75 mg/ml and 77.5 mg/ml, respectively. Vancomycin was found to possess high inhibitory activity against bacteria under investigation, with MIC values of 2.5 µg/ml.

Effect of SH decoction on bacterial adhesion. From the results of the spread plate method (Fig. 1), 1/2MIC SH, 1MIC SH and TSB-vancomycin groups could significantly inhibit the bacterial adhesion on the titanium surface ($P < 0.01$), compared with TSB, 1/8MIC SH and 1/4MIC SH groups. While among TSB, 1/8MIC SH and 1/4MIC SH group of *ATCC* 35984, no significant difference were observed ($P > 0.05$). For *ATCC* 43300, compared with TSB group, both 1/4MIC SH and 1/8MIC SH groups also inhibit the bacterial adhesion on the titanium surface ($P < 0.05$); while no significant difference between 1/4MIC SH and 1/8MIC SH groups were found ($P > 0.05$). We also observed statistically significant difference in the quantitative analysis of bacterial adhesion between SH groups under 1/4, 1/2 and 1MIC concentrations, which was more obvious with the increase of concentration of SH decoction ($P < 0.01$). On the other hand, vancomycin at MIC had the strongest inhibitory effect on the bacterial adhesion on the titanium surface, compared with other groups ($P < 0.01$).

Further, we performed SEM (Fig. 2) 6 h after incubation with bacteria and showed that scattered bacterial colonies were observed on the titanium surface in the 1/2MIC SH, 1MIC SH and vancomycin group. However, the bacteria in the TSB, 1/8MIC SH and 1/4MIC SH group obviously clumped together. For SH groups with 1/4, 1/2 and 1MIC concentrations, the bacterial adhesion on the surface of titanium disc became more obvious with the increase of dilution of SH decoction. Meanwhile, the number of adherent bacterial colony on the titanium surface in vancomycin group was the least in all groups. SEM qualitative analysis is consistent with the conclusion of the quantitative data in Fig. 1.

Antibiofilm activity to bacterial strains of SH decoction. As shown in Fig. 3, compared with the TSB and 1/8MIC SH groups, the OD values of 1/4MIC SH, 1/2MIC SH, 1MIC SH and vancomycin groups were significantly decreased ($P < 0.01$), while there was no significant difference between TSB and 1/8MIC SH groups ($P > 0.05$). For the SH groups in the two strains, the OD values decreased with the increase of SH concentration, and the difference among the 1/8MIC, 1/4MIC, 1/2MIC and 1MIC SH groups was statistically significant ($P < 0.01$). For 1/4, 1/2 and 1 MIC of SH solution, the OD value was significantly decreased, among which the OD values of the 1MIC SH groups were lower than 0.120, indicating no biofilm formation on the titanium surface. Though the OD value of 1/2MIC SH group was significantly decreased; it was between 0.120 and 0.240, suggesting that there was still mild biofilm formation¹⁵. For 1/4MIC SH group, the OD value was more than 0.240, indicating the biofilm formation¹⁵. The OD value of vancomycin group was lower than that of 1MIC group ($P < 0.01$), which also indicated that there was no biofilm formation.

CLSM of three-dimensional structure of bacterial biofilms on the titanium surface is shown in Fig. 4. After culture for 24 h, a large number of bacterial colonies were clustered to form a dense biofilm structure on the titanium surface in the TSB and 1/8MIC SH groups, displaying high-intensity green fluorescence. However, with the increase of the SH concentration, the intensity of green fluorescence gradually decreased, suggesting that the bacterial biofilm formation was also gradually inhibited. For *ATCC* 35984, high-intensity green fluorescence was still visible in 1/4MIC SH group, indicating the existence of severe biofilm formation; While in 1/4MIC SH group of *ATCC* 43300, in 1/2MIC SH groups of two bacterial strains, low-intensity green fluorescence was shown, indicating that there was still mild biofilm formation on the titanium surface. In the 1MIC SH and vancomycin groups, only scattered green fluorescence of the bacterial colonies were seen, showing no biofilm formation.

Effect of SH decoction on biofilm formation at different incubation time-point. CV staining (Fig. 5) showed that the OD value of biofilm had an increasing trend in TSB and 1/8MIC SH groups with the increase of incubation time, suggesting the strains adhered to the titanium surface and gradually formed biofilm; from 6 h to 24 h, we observed significant differences of the OD values between any two time-points ($P < 0.01$), while no statistical difference of OD value was found for *ATCC* 35984 ($P > 0.05$) and statistical difference of OD value was found for *ATCC* 43300 ($P < 0.01$) between 24 h and 48 h. For the 1/4MIC SH group of *ATCC* 35984, the OD values increased and difference between any two time-point were found from 6 h to 18 h ($P < 0.01$), then no difference from 18 h to 48 h ($P > 0.05$); for *ATCC* 43300, the OD values of 1/4MIC SH group increased and difference between any two time-point were statistically significant from 6 h to 48 h ($P < 0.05$). On the contrary, the OD values from 6 h to 48h in the 1/2MIC SH, 1MIC SH and vancomycin groups were stable, and no obvious difference was found between any two time-points for 1MIC SH and vancomycin groups ($P > 0.05$). At different time point, the OD values in the TSB, 1/8MIC SH, 1/4MIC SH groups were higher than that in 1/2MIC SH, 1MIC SH and vancomycin groups ($P < 0.01$), and significant differences were also found among the 1/8MIC, 1/4MIC, 1/2MIC, 1MIC SH and vancomycin groups ($P < 0.05$).

SH effects on the expression of gene *icaAD* and *icaR*. As shown in Fig. 6, the expression level of *icaAD* was not significantly different between the TSB and 1/8MIC SH groups ($P > 0.05$). The expression level of *icaA* and *icaD* in the 1/4 MIC, 1/2MIC, and 1MIC SH groups decreased compared with that of the TSB and 1/8MIC groups ($P < 0.01$). In the SH groups, the *icaA* expression level showed a gradual decrease with the increase of SH concentration, showing statistically significant differences ($P < 0.01$). Further, no differences of *icaD* were found among the 1/4MIC, 1/2MIC, and 1MIC SH groups ($P > 0.05$). For *icaR* expression level, there were no statistically significant differences between the TSB and 1/8MIC SH groups or the 1/2MIC and 1MIC SH groups ($P > 0.05$). Compared with the TSB and 1/8MIC SH groups, the 1/4MIC, 1/2MIC and 1MIC SH groups showed high expression level ($P < 0.01$). The expression levels of *icaR* were also different among the 1/8MIC, 1/4MIC and 1/2MIC (1MIC) SH groups ($P < 0.01$).

Discussion

The clinical treatment of postoperative implant-related infections still remains as a challenge for orthopedic surgeons, which is mainly related to the formation of bacterial biofilms at the infected site³. Biofilms are communities of bacteria that are attached to a surface and surrounded by a self-produced matrix, consisting of polysaccharide as well as proteins, nucleic acids, lipids, and humic substances³. Due to the formation of biofilms, intravenous antibiotics often fail to completely eliminate pathogenic bacteria of orthopedic implant-related infections. Even if the local symptoms are eliminated in a short time, the infection will recur again when the patient's immunity is reduced¹⁶. In addition, biofilms also increase bacterial resistance to antibiotics¹⁷. Therefore, the inhibition of biofilm formation and the killing of antibiotic-resistant bacteria are the key methods for effective treatment of the refractory infections related to the orthopedic implants. For these reasons, in addition to thorough debridement, intravenous use of sensitive antibiotics is necessary for the clinical treatment of infections. However, unfortunately, multidrug-resistant bacteria are often found in the bacterial culture of clinical orthopedic infection wounds, and based on the antibiotic sensitivity tests, vancomycin has often become the first choice for clinicians¹⁸. Studies have shown that only a small portion of intravenous vancomycin can reach bone and joint tissues, and even less penetrates into bone tissues at the site of osteomyelitis; thus, vancomycin may not eliminate multidrug-resistant pathogens in biofilms¹⁹. In addition, the application of vancomycin has led to the emergence of vancomycin resistant *Staphylococcus* strains²⁰. Linezolid is another novel antibiotic that has been found to be useful in the treatment of vancomycin failed MRSA infections, but safety concerns have limited its widespread use²⁰.

Due to the aforementioned defects in antibiotics for the treatment of biofilm-related infections caused by multidrug-resistant pathogens, seeking other treatment methods for refractory orthopedic biofilm-related infections is urgent and necessary. One such treatment strategy is the external application of the TCM, which has been used in the Chinese population to prevent and treat orthopedic infections for long time, and many prescriptions have been proved to be therapeutically effective⁸⁻¹⁰. According to the literature review, the external application of TCM has beneficial properties such as anti-infection and local immune regulation in the infected wound, which has advantages in the control of acute and chronic local orthopedic infections. For local infections of extremities caused by multi-antibiotic-resistant bacteria with exposure of bone and implants, based on the TCM theory of "simmer pus promoting the regeneration", we have adopted SH decoction to directly soak and wash the infected wounds on the basis of the long-term clinical results of a large number of cases indicating that the local infection of the limbs was obviously controlled. Additionally, we observed that the swelling of the tissue subsided, pus and necrotic tissue decreased, which provided better tissue conditions for reoperation and wound healing. This decoction contains *Scutellaria baicalensis* Georgi, *Coptidis rhizoma*, *Cortex Phellodendri chinensis* and so on, which are heat-clearing and detoxifying drugs. Modern medical research have shown that these Chinese herbs had a broad-spectrum antibacterial and bacteriostatic effects, and no bacterial resistance was found in the combined application. The clinical application of SH prescription can play the role of anti-bacterial and anti-inflammatory in the wound, thus achieving the purpose of eliminating infections⁸⁻¹⁰.

This study investigated the *in vitro* effect of SH decoction on adhesion and biofilm formation of bacteria on the titanium surface, and explored the potential value of this prescription as an effective method to treat orthopedic infections. Since the majority of orthopedic implant-related infections are caused by *S. epidermidis* and *S. aureus*²¹. Therefore, we selected the ATCC 35984 and ATCC 43300 to understand biofilm formation in this study. Previous research have demonstrated that biofilm formation proceeds in two phases: primary attachment of *staphylococcal* cells on a biomaterial, which is followed by bacterial accumulation in multiple layers and glycocalyx formation leading to a mature biofilm, the attachment of *Staphylococcus* to solid surfaces is strongly seen as an essential step in the formation of biofilm^{3,6}. If the primary bacterial adhesion is interfered, then the biofilm formation could be prohibited consequently^{3,14}. The results in this study showed that SH decoction could inhibit the bacterial adhesion on the titanium surface, and there was a trend that the inhibition effect was more obvious with the increase of dilution concentration of SH decoction. The results also showed that SH decoction could inhibit the formation of dense structural biofilms of the antibiotic-resistant *staphylococcus* on the titanium surface by either quantitative or qualitative analysis. Specifically, the bacterial strains cannot form biofilms when incubated with the 1MIC or 1/2MIC of SH decoction, only showing loose adhesion of the bacterial colonies. Although biofilm was formed at 1/4 MIC, its OD values were obviously less than that of TSB group. At 1/8MIC of SH decoction, biofilm formation was not inhibited compared to the TSB control group. It can be seen from above analysis that SH decoction could inhibit the biofilm formation on the titanium surface, which is dependent on the dilution concentration of the SH decoction.

The results in the work showed that SH decoction with high concentration has an obvious inhibition of the bacterial adhesion and biofilm formation on the titanium surface. However, the mechanism that allows the inhibition of biofilm formation is not clear. Many studies have shown that polysaccharide intercellular adhesin (PIA) is the primary determinant of the accumulation phase of *staphylococcal* biofilm formation²¹⁻²³. Although bacteria with PIA deficiency have been able to adhere to the surface of medical implants at the initial stage of biofilm formation, the ability of a large number of colonies to gather and form biofilm at the later stage were greatly reduced, and even bacterial biofilms could not be formed^{3,6,23}. It has been reported that the *ica A* and *ica D* partially overlaps in sequence and they together mediate the synthesis of N-acetylglucosamine using UDP-N-acetylglucosamine as a substrate, which composes β -1,6-linked homoglycan to synthesize PIA²⁴. It can be seen that the co-expression of the functional *ica A* and *ica D* gene can catalyze the synthesis of PIA, and the down-regulation of *ica AD* expression leads to the reduced activity of N-acetylglucosaminyl transferase, which also leads to reduced amount of PIA²⁴⁻²⁶. In addition, studies have shown that the complex gene regulation system of *staphylococcus* may ultimately influence the biofilm formation by regulating the expression of *ica* operon^{23,26}. Therefore, in order to verify the role of *ica* operon in the inhibition of bacterial biofilm formation on the titanium surface by SH decoction, ATCC 35984 with property of positive biofilm formation and *ica AD* gene expression was selected in this study¹¹. The expressions of *ica AD* were detected by real-time fluorescence quantitative PCR, and the results showed that during the process of biofilm formation, the expression level of *ica A* and *ica D* genes in the biofilm and culture medium was

inhibited by the SH decoction at different dilution concentrations, and the inhibition became more obvious with the increase of SH concentration. In addition, the expression of *ica* operon is negatively regulated by its upstream inhibitory gene *ica R*²⁶. In our study, we found that SH decoction could not only down-regulate the expression of *ica AD*, but also up-regulate the expression level of *ica R* in a concentration dependent manner. Therefore, it could be inferred that the SH decoction activates *ica R* to inhibit the transcription of *ica AD*, thus leading to the reduced synthesis of PIA and formation of biofilm on the titanium surface.

Conclusion

It is evident that SH decoction can inhibit biofilm formation of antibiotic-resistant *Staphylococcus* on the titanium surface based on our *in vitro* results. Further, there appears to be a concentration dependent relationship between SH decoction and biofilm formation. The mechanism may be that the SH decoction activates *ica R* to inhibit the transcription of *ica AD*, thus, leading to reduced synthesis of PIA and prevention of biofilm formation.

Declarations

Acknowledgements

This study was supported by the NSFC-Henan Joint Fund (No.U1804189) and Henan science and technology research project (No.162102310369)

Author Contributions

S.E.Z. wrote the manuscript; P.Z.W. and X.T.S. analyzed the raw data; H.L.T. conceived the study, participated in the design and coordination. All authors reviewed the manuscript.

Competing Interests

The authors declare no competing interests.

Additional Information

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Tables

Table 1
The composition of SHJD decoction.

Chinese name	Latin name	Part used	Proportion (g)
Huang Qin	Scutellaria baicalensis Georgi	Root	25
Huang Lian	Coptidis rhizoma	Root	25
Huang Bai	Cortex Phellodendri chinsis	Bark	25
Ku Shen	Sophora flavescens	Rhizome	20
Jin Yin Hua	Lonicera Japonica	Bud	20
Lian Qiao	Forsythia suspensa	Fruit	20
Pu Gong Ying	Taraxacum mongolicum Hand.-Mazz.	Root	20

Table 2
Primers used in this study

Gene	Gene description	Primer sequence (5'-3')	
ica A	Intercellular adhesion A	Forward	AACAAGTTGAAGGCATCTCC
		Reverse	GATGCTTGTTTGATTCCCT
ica D	Intercellular adhesion D	Forward	ATCGTTGTGATGATTGTTTA
		Reverse	GATATGTCACGACCTTTCTT
ica R	Intercellular adhesion R	Forward	CCATTGACGGACTTTACCAG
		Reverse	CAAAGCGATGTGCGTAGGA
16S rRNA	Normalizing internal standard	Forward	TCGTGTCGTGAGATGTTGGGTTA
		Reverse	GGTTTCGCTGCCCTTTGTATTGT

Table 3
MIC and MBC of SH against bacterial strains. -: no bacteria growth; +: bacteria growth.

SHJD/(mg/ml)	ATCC 35984		ATCC 43300	
	MIC	MBC	MIC	MBC
4.84	+	+	+	+
9.69	+	+	+	+
19.38	+	+	+	+
38.75	-	+	-	+
77.5	-	-	-	-
155	-	-	-	-

Figures

Figure 1

Attachment assay of bacterial strains on titanium surface. For ATCC 35984, compared with TSB, 1/8MIC SH and 1/4MIC SH group, *P < 0.01. For ATCC 43300, compared with TSB group, # P < 0.05; compared

with TSB, 1/8MIC SH and 1/4MIC SH group, $P < 0.01$.

Figure 2

SEM of bacterial adhesion on the titanium surface. Compared with TSB, 1/8MIC SH and 1/4MIC SH groups, 1/2 and 1MIC SH groups can obviously inhibit bacterial adhesion (1000×). (A) TSB group. (B) 1/8MIC SH group (C) 1/4MIC SH group. (D) 1/2MIC SH group. (E) 1MIC SH group. (f) Vancomycin group.

Figure 3

CV staining of bacterial biofilms under different culture conditions. Compared with the TSB and SH groups, * $P < 0.01$.

Figure 4

CLSM of biofilm formation on the titanium surface. High-intensity fluorescence was seen in the TSB, 1/8MIC SH, and 1/4 MIC SH groups, and low-intensity fluorescence was seen in the 1/2MIC SH, 1MIC SH, and vancomycin groups (50×). (A) TSB group. (B) 1/8MIC SH group. (C) 1/4MIC SH group. (D) 1/2MIC SH group. (E) 1MIC SH group. (F) Vancomycin group.

Figure 5

CV staining at different culture time. Compared with TSB and 1/8MIC SH groups, * $P < 0.01$; Compared with 1/4MIC SH group, # $P < 0.05$.

Figure 6

Analysis of *ica* gene expression (PCR). Compared with TSB and 1/8MIC SH groups, * $P < 0.01$, ** $P < 0.01$.