



Smart Chimeric Lysin ClyC Loaded Alginate Hydrogel Reduces *Staphylococcus aureus* Induced Bone Infection

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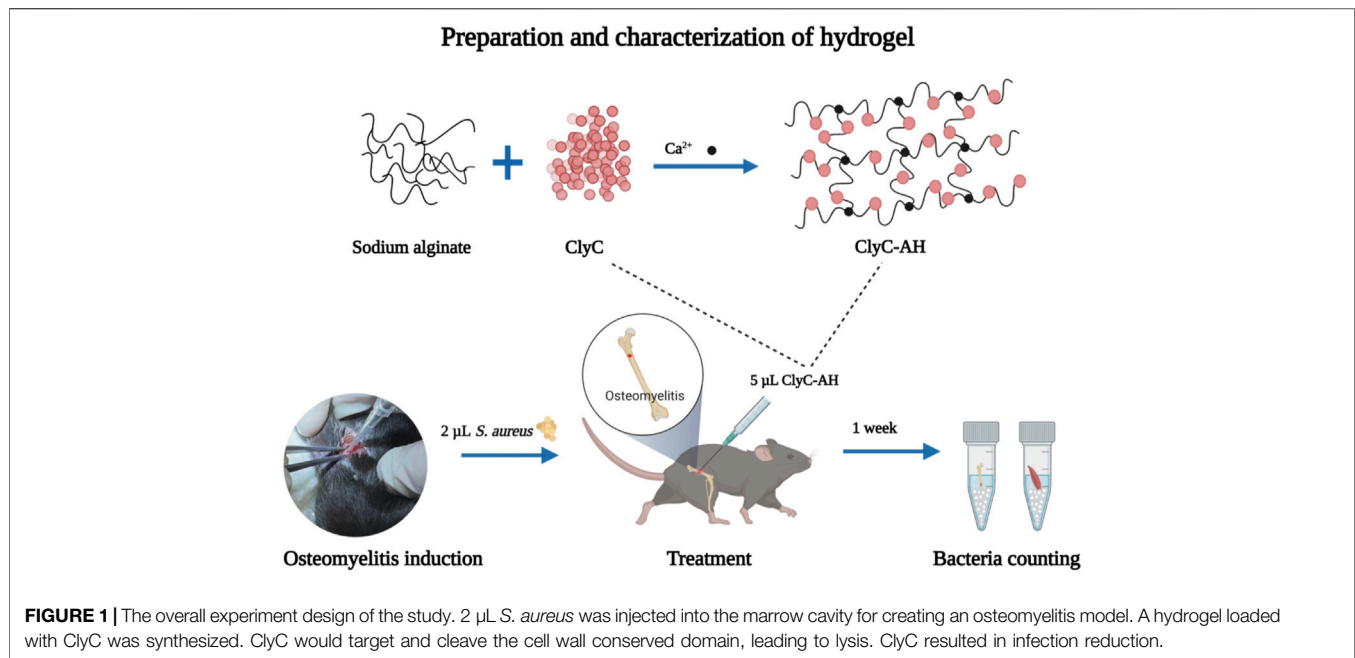
Staphylococcus aureus (*S. aureus*) is the most common cause of hospital and community-acquired infections. The current clinical treatment is limited by the emergence of drug-resistant strains. We previously developed a chimeric ClyC that effectively inhibited *S. aureus* strains. Nonetheless, an efficient delivery system to provide sustained release of ClyC to infected site is needed. Thus, we engineered a chimeric ClyC loaded alginate hydrogel (ClyC-AH) to improve the therapeutic outcomes against *S. aureus*. ClyC-AH retained the stability and activity of ClyC while providing a sustained release of ClyC and a continuous antibacterial effect against *S. aureus*. Compared to ClyC alone, the use of ClyC-AH was relatively safe, as there was no significant cytotoxicity to BHK-21 cells at a ClyC concentration $\leq 250 \mu\text{g/ml}$. Furthermore, in a *S. aureus* infected mouse model of osteomyelitis, ClyC-AH reduced bacterial burden in the femur and surrounding tissues, with a reduction of $2 \log_{10}$ (CFU/ml) in viable bacterial number. Based on these results, hydrogel-delivered chimeric lysin ClyC provides a promising future in the *S. aureus* targeting therapy.

Keywords: *Staphylococcus aureus*, MRSA, osteomyelitis, anti-bacterial activity, bacteriophage lysin, alginate hydrogel

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive pathogen that primarily colonizes on the nostrils, skin, and mucosal membranes of human (Lowy, 1998; Becker and Bubeck-Wardenburg, 2015). *S. aureus* is the main conditional pathogen contributing to hospital and community-acquired infections, leading to infectious diseases such as osteomyelitis (Favero, 2003; Lindsay and Holden, 2004).

Osteomyelitis is a challenging infectious medical condition for orthopaedic surgeons (Lew and Waldvogel, 2004; Maffulli et al., 2016). Approximately 44% of the osteomyelitis are caused by *S. aureus* (Kremers et al., 2015), through hematogenous infection or the spread of a contiguous source. Treatment for osteomyelitis mainly include use of antibiotics and surgical debridement (Conterno and Turchi, 2013). However, *S. aureus* could directly invade and colonize the osteocyte lacuno-cannicular network (Maffulli et al., 2016; Urish and Cassat, 2020; Gimza and Cassat, 2021), which might bring difficulty to anti-infectious treatment. In addition, the pathogenic characteristics of *S. aureus*, including biofilm formation, transmission of drug-resistant genes, and intracellular survival (Pantosti et al., 2007; Davies and Davies, 2010; Munita and Arias, 2016), further limit the therapeutic



outcomes. Thus, finding novel antibacterial agents against *S. aureus* has captured the attention of scientists in recent years.

Endolysins are enzymes encoded by bacteriophages, which specifically target and degrade the peptidoglycan externally, to cleave bacteria (Haddad Kashani et al., 2018; Gondil et al., 2020b). Endolysins have strong bactericidal activity and low susceptibility to resistance. Thus, they are emerging as a viable alternative to antibiotics (Abdelrahman et al., 2021). Some endolysins are successfully applied in clinical trials. CF-301 has showed impressive results in treating patients with *S. aureus* bloodstream infections and endocarditis. Furthermore, CF-301 has been used in phase 3 clinical trials, indicating the potential effect of endolysins on bacterial infections (Jun et al., 2017; Fowler et al., 2020).

ClyC, a novel chimeric lysin, has specificity and robust killing efficacy against drug-resistant and susceptible *S. aureus* and other *staphylococcal*, the effect of which could be further enhanced with calcium. Also, ClyC protected mice from lethal systemic *S. aureus* infection (Li et al., 2021). However, the effect of endolysins are limited by its instability and short half-life *in vivo*, as well as the cytotoxicity tested *in vitro* (Resch et al., 2011; Seijnsing et al., 2018). Thus, a suitable delivery mechanism is required to prevent ClyC from degradation, enhance the biocompatibility, and facilitate ClyC entry into the infection sites (Pinto et al., 2021).

Alginate is a linear copolymer derived from brown seaweed that contains repeating units of β -1,4-linked D-mannuronic acid and L-guluronic acid residues. Alginate hydrogels are widely used in wound dressing and bone and cardiac tissue engineering due to their biocompatibility, low cytotoxicity, low immunogenicity, and biodegradability (Ruvinov and Cohen, 2016; Varaprasad et al., 2020). They are ideal materials for the delivery of protein drugs because they can be prepared under mild pH and temperature conditions (Lee and Mooney, 2012). Antibacterial agents have been encapsulated in alginate hydrogel in order to target specific pathogens and reduce infections. LysMR-5 incorporated into alginate-chitosan

nanoparticles recently exhibited enhanced bactericidal activity against *S. aureus in vitro*, but its stability was not assessed in that study (Kaur et al., 2020).

In this study, we designed ClyC loaded alginate hydrogel (ClyC-AH) to treat osteomyelitis caused by *S. aureus* (Figure 1). Through the encapsulation process, the stability and bactericidal activity of endolysin were optimized. We hypothesized that the ClyC-AH could sustainably release ClyC, which process the property of ClyC remained stable and the cytotoxicity reduced. Furthermore, we tested the efficacy of ClyC-AH in a *S. aureus* infected osteomyelitis mouse model. The change of bacteria colony forming unit (CFU) was tested to demonstrate the therapeutic potential of the ClyC-AH.

MATERIALS AND METHODS

Preparation of ClyC-AH

The chimeric lysin, ClyC, was expressed as described previously (Li et al., 2021). Concentration of ClyC was determined by BCA assay and visualized by SDS-PAGE. The ClyC protein solution was freeze-dried and then stored at -80°C until use. An optimized ClyC-AH formulation was prepared using a one-step procedure at room temperature as proposed by Deng and Feng (Deng et al., 2015; Feng et al., 2020), with slight modification. Briefly, sodium alginate was dissolved in distilled water and magnetically stirred for 3 h until it completely dissolved. Freeze-dried ClyC was mixed with 0.68% calcium chloride solution (Hushi, Shanghai, China). Then, the mixture was added into 1.5% alginate solution at a ratio of 1:1 (v/v) and homogenized. The gelation time was measured by the inversion tube method at room temperature. In brief, 400 μL ClyC-AH was taken in a 1.5-ml Eppendorf tubes and incubated at room temperature. The gelation time was confirmed by inverting the tube every 5 s until the gel stopped flowing. Each solution was

filtered separately through a sterile 0.22- μm membrane into a sterile container.

Scanning Electron Microscopy

Hydrogel alone and ClyC-AH were separately immersed in Tris buffer at room temperature until reached swelling equilibrium, cut into sections (10 mm \times 7 mm \times 5 mm). The samples were lyophilized using the LyoBeta 6 PL (Telstar, Spain) instrument, coated with gold (Hummer VI; Technic Inc., Anaheim, CA, United States). The microstructure of the sample was evaluated using the SEM (SU8010, Hitachi, Japan) at an accelerating voltage of 3 kv.

Cytotoxicity Test

The toxicity of ClyC released from hydrogel was evaluated using the CCK-8 assay. Alginate hydrogels with different ClyC concentrations (0, 31.25, 62.5, 125, 250, 500, and 1,000 $\mu\text{g}/\text{ml}$) were incubated in equal-volume Dulbecco's modified Eagle's medium (DMEM; Sigma, United States) at 37°C. After 24 and 48 h, the whole medium was collected. To investigate the cytotoxicity of the ClyC in the harvested medium, Baby hamster Syrian kidney (BHK)-21 cells were used. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C under 5% CO_2 . BHK-21 cells were seeded into 96-well at a density of 1×10^4 cells/well. After 24 h, the cell culture medium was exchanged with the harvested medium (100 μL) and the same concentration of soluble ClyC as a control. After incubation for 24 h, the cell viability was measured by CCK-8 assay. The normal cell culture medium without ClyC was used as a positive control (PC), while medium without ClyC and BHK-21 cells served as a negative control (NC). The relative cell viability was determined by using the formula: %cytotoxicity = $[1 - (\text{OD}_{\text{sample}} - \text{OD}_{\text{NC}}) / (\text{OD}_{\text{PC}} - \text{OD}_{\text{NC}})] \times 100\%$.

Determination of the Amount of ClyC Released From Alginate Hydrogels

To investigate the release amount of ClyC from alginate hydrogels, the ClyC-AH (250 $\mu\text{g}/\text{ml}$, 400 μL) was incubated in Tris buffer (100 μL in 1.5-ml Eppendorf tubes) at 37°C. At predesignated time intervals (1, 3, 6, 10, 14, 24, 36, 48, and 72 h). At each timepoint, the supernatant was sampled, and the same volume of fresh Tris buffer was added. The amount of released ClyC from alginate hydrogels at each time point was determined using the BCA method and calculated based on the standard curve. The structural integrity of ClyC after release was examined using SDS-PAGE analysis. The ClyC released from hydrogel was suspended in SDS-PAGE loading buffer, destabilized by boiling for 10 min, processed and analyzed by SDS-PAGE, following standard protocol. Free native ClyC was also processed similarly and analyzed along with the test sample as a reference.

The Anti-Bacterial Activity Assays of ClyC Released From Alginate Hydrogels

The bacteria strain used in the study was *S. aureus* T23. The inoculum was prepared by culturing *S. aureus* T23 overnight in lysogeny broth (LB) medium with shaking at 37°C, washing three

times in Tris buffer, by centrifugation, and adjusting the optical density at OD_{600} (Synergy H1). A preliminary assessment of the anti-bacterial effect of ClyC released from hydrogel was conducted *via* turbidity reduction experiment and inhibition zone method. For turbidity reduction experiment, the decrease of samples in OD_{600} was monitored by a Synergy H1 microplate reader every 1 min for 10 min at 37°C. For the inhibition zone method, 10 μL ClyC and ClyC-AH were spotted onto agar plates overlaid with T23 and incubated overnight at 37°C. Bactericidal activity was assessed by clear zones on the agar plates. Comparison of bactericidal activity of different timepoint sample was done by measuring colony count. Samples from each timepoint (50 μL) incubated with 50 μL T23 inoculum at 37°C for 1 h, serial dilution, and plating on lysogeny broth agar. Free ClyC solutions (125 $\mu\text{g}/\text{ml}$) incubated at 37°C were also prepared at each timepoint.

In vivo Treatment of Osteomyelitis With ClyC-AH

This study was approved by the Ethics Committee for Animal Research, Wuhan University, China (No. 2019A79). C57BL/6 mice were housed in 12-h light/dark cycles and freely provided food and water. The mice were assigned to four groups (bacteria infected, bacteria infected with hydrogel alone, bacteria infected with soluble ClyC alone, and bacteria infected with ClyC-AH groups; $n = 5$ or 6 per group). Briefly, after anesthetized, the skin on the right knee of mice was shaved and sterilized with 75% alcohol. A lateral incision was made, and the muscle was bluntly dissected to expose the femur. The proximal end of the femur was perforated using a high-speed drill with a 0.5-mm sterile round bur (Cassat and Skaar, 2013; Klopfenstein et al., 2021). T23 (3×10^6 cell CFU/mL in 2 μL of PBS) was inoculated into the medullary cavity of the femur using 10 μL -pipet tips and then injected 5 μL of different treatments. For animals receiving hydrogel alone, the hydrogel was pipetted over the hole to polymerize. For animals receiving soluble ClyC, the concentration of ClyC was equal to that of ClyC-AH. Then the wound was double-stitched by surgical silk suture.

After 1 week, all mice were sacrificed. The skin on the right hind leg was disinfected with 75% alcohol. After making a lateral incision, the femur and surrounding tissue were separated, weighed, and placed in 1.5-ml microcentrifuge tubes (with saline). Following dissection and removal, all samples were maintained on ice. The tissue and femur samples were subsequently homogenized using the MD100 by bead beating (5,000 rpm, 20 s) (NewZongKe, China) (Johnson et al., 2018). Water bath sonication was used to create single-cell bacterial suspensions after homogenization (sonicate 10 min, vortex 30 s). Homogenates were serially diluted, plated on Baird-Parker agar, and incubated overnight at 37°C. Colonies were accounted, normalized to sample weight, and transformed using the formula $\text{CFU} = \log_{10} (1 + X)$ to avoid negative values.

Statistics Analysis

Experimental data were expressed as mean \pm standard deviation. Statistical analyses were conducted using Prism v

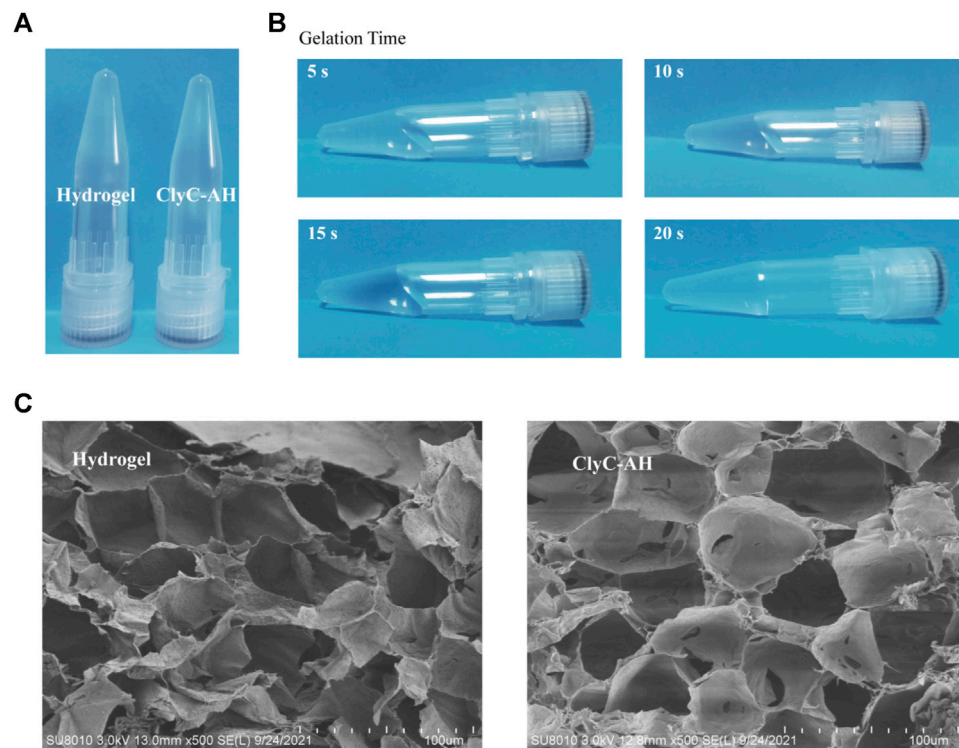


FIGURE 2 | Characterization of the ClyC-AH. **(A)** Images of the hydrogel and ClyC-AH at room temperature; **(B)** Gelation time showing the solution-gel transition of ClyC-AH; **(C)** Surface morphologies of hydrogel and ClyC-AH by SEM.

8.0.2 (GraphPad Software, United States). Statistical comparisons between two groups were made with Student's *t* test. ANOVA for multivariate parametric data with a Tukey's post hoc test or a Kruskal–Wallis test with Dunn's post hoc test for nonparametric data. *p*-value of <0.05 was considered as statistically significant.

RESULT

Characterization of the ClyC-AH

ClyC-AH were prepared simply by mixing 1.5% alginate solution and 0.68% calcium chloride solution-containing ClyC, which was the most common method to prepare the hydrogel from the sodium alginate solution (Lee and Mooney, 2012). From a macroscopic view, there was no significant difference between ClyC-AH and alginate hydrogel without ClyC, which were both transparent and colorless (Figure 2A). The morphology of the samples was observed using SEM. The surface morphology of hydrogel alone and ClyC-AH was similar, which showed porous structure with good continuity. There were tiny pores observed in the structure which was thought to be beneficial for the sustain ClyC release (Figure 2C). The gelation time of ClyC-hydrogel was about 20 s, short but sufficient for injection into the lesion (Figure 2B). Meanwhile, it was conducive for the rapid fixation of ClyC-AH in the lesion.

ClyC-AH Reduced ClyC Cytotoxicity

Considering that high concentration of ClyC could cause cytotoxicity in the local infection, we loaded ClyC into alginate hydrogels. The results showed that the cytotoxicity of ClyC-AH was reduced compared with soluble ClyC (Figure 3). At 24 h after incubation, with the concentration of ClyC up to 250 µg/ml, the cytotoxicity of ClyC-AH showed no significant difference among different concentrations. Soluble ClyC alone had toxic effect on BHK-21 cells. Compared with soluble ClyC, ClyC-AH significantly improved cell viability. It could be explained by the slow release of ClyC from alginate hydrogels. The results suggested that the blank and loaded 250 µg/ml ClyC-AH were not cytotoxic to BHK-21 cells.

In vitro Release of ClyC From Alginate Hydrogels

The amount of ClyC released from the alginate hydrogels are showed in Figure 4A. After 72 h, the cumulative released amount of ClyC from alginate hydrogels was approximately $23 \pm 0.45\%$. To test the structural integrity of ClyC released from the alginate hydrogels, SDS-PAGE analysis was used and showed a single band corresponding to the molecular weight of free ClyC, as could be seen in Figure 4B. No additional band below the ClyC from the alginate hydrogels band, proving that ClyC was not

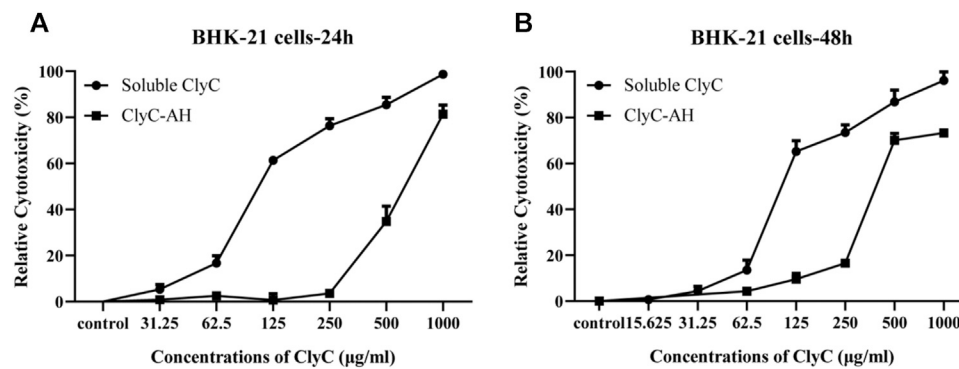


FIGURE 3 | Cytotoxicity of ClyC-AH and free ClyC to BHK-21 cells. Firstly, DMEM incubated with ClyC-AH (0, 31.25, 62.5, 125, 250, 500, and 1,000 µg/ml) for 24 h (A) and 48 h (B). Then cells were co-cultured with incubated DMEM and different concentrations of free ClyC (0, 15.625, 31.25, 62.5, 125, 250, 500 and 1,000 µg/ml) for 24 h. The relative viability was calculated by the change of OD₄₅₀ by CCK-8 assay.

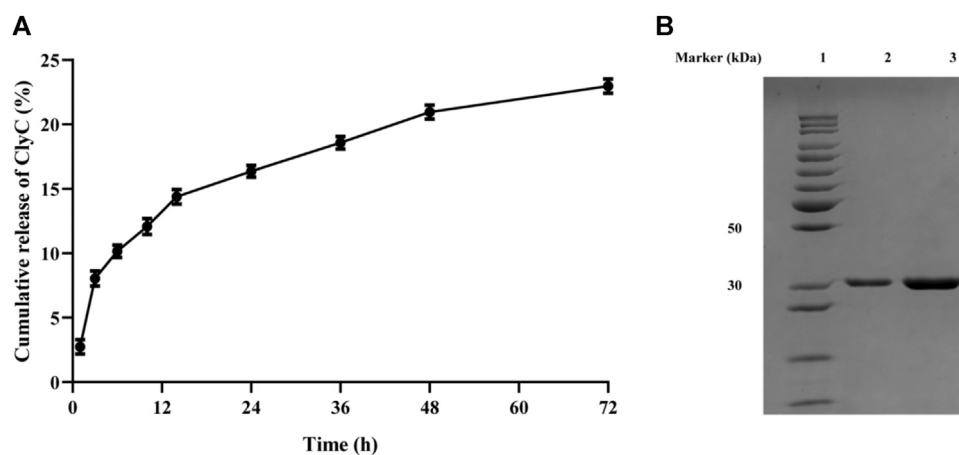


FIGURE 4 | *In vitro* release of ClyC from hydrogel at 37°C in Tris buffer. (A) The curve of percent released ClyC at different time intervals. Mean ± SD, $n = 3$; (B) SDS-PAGE of free ClyC and ClyC released from hydrogel to determine the structural integrity of endolysin after encapsulation. Lane 1: Standard Protein Marker. Lane 2: ClyC released from the hydrogel. Lane 3: ClyC free native ClyC.

hydrolyzed or degraded during the process of hydrogel gelation and release.

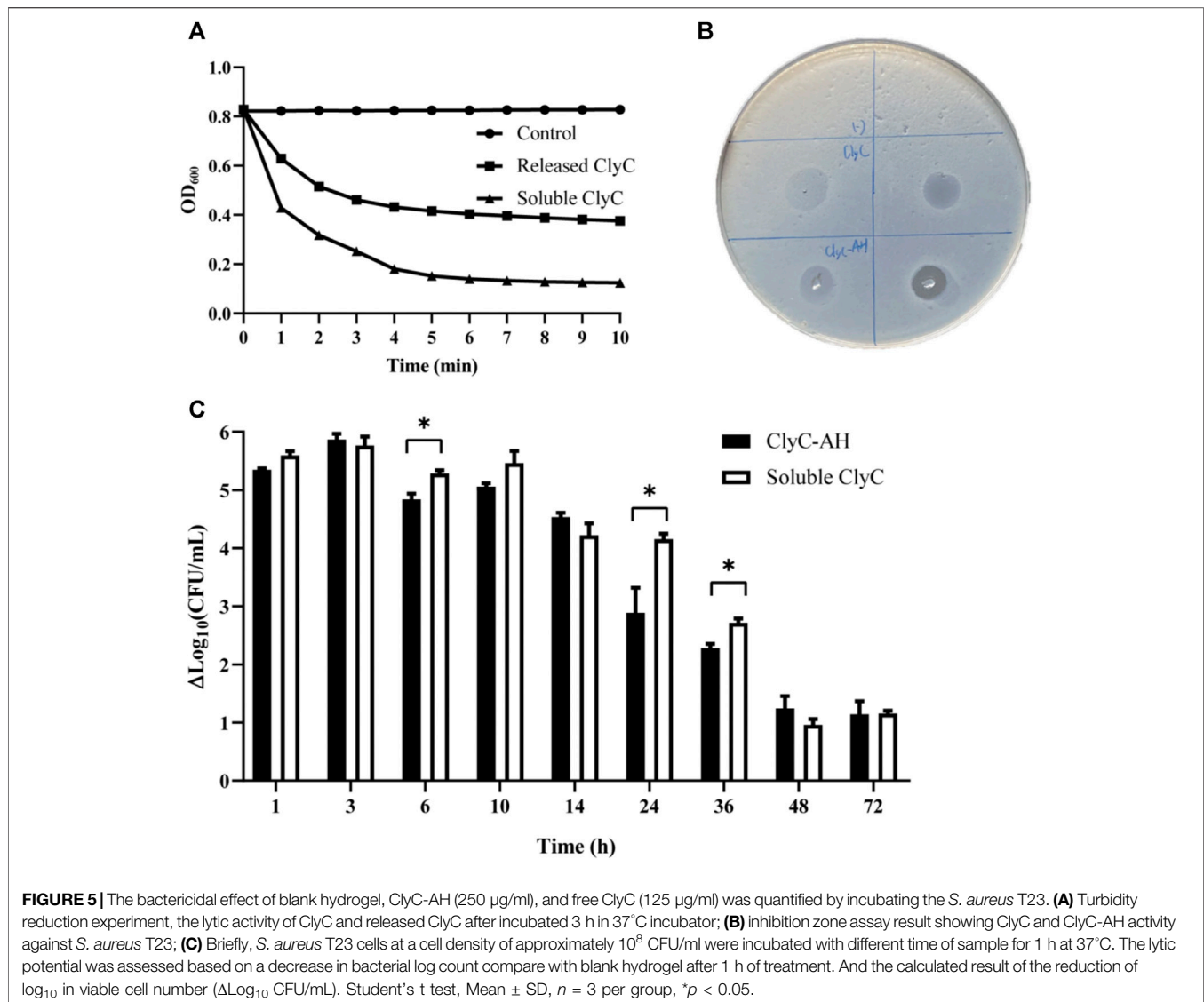
The Hydrogel Maintains ClyC Antibacterial Activity *in vitro*

As reported before, ClyC had highly anti-bacterial against *S.aureus* and showed enhanced bactericidal activity in the presence of calcium. In the turbidity reduction experiment, the OD₆₀₀ values of T23 bacterial suspensions reduced rapidly (Figure 5A). In support, clear zones could also be seen in the plates after dipping ClyC and ClyC-AH on the lawn of *S. aureus* (Figure 5B), which showed that the released ClyC retained bactericidal activity. The bactericidal activity against *S.aureus* T23, by ClyC released from the hydrogel after different time intervals, was compared. The results showed that the ClyC released from hydrogel had high bactericidal activity within

24 h. After incubation in 37°C incubator for 24 h, ClyC could lead to a reduction of 2.89 log₁₀ in viable cell numbers. Moreover, after incubation for 72 h, there was still a 1.14 log₁₀ reduction in viable cells, indicating a therapeutic effect for acute infection (Figure 5C).

In vivo Treatment of Osteomyelitis With ClyC-AH

The osteomyelitis mouse model was established to evaluate the ability of ClyC-AH to inhibit *S. aureus* infection *in vivo*. ClyC-AH was injected into the medullary cavity using 10 µL-pipet tips and subsequently transformed into hydrogels which allow stable injection into the cavity without leakage. The number of bacteria CFU in the femur and surrounding tissue was measured 7 days after surgery to evaluate the severity of osteomyelitis. Both the infection-only and hydrogel-only



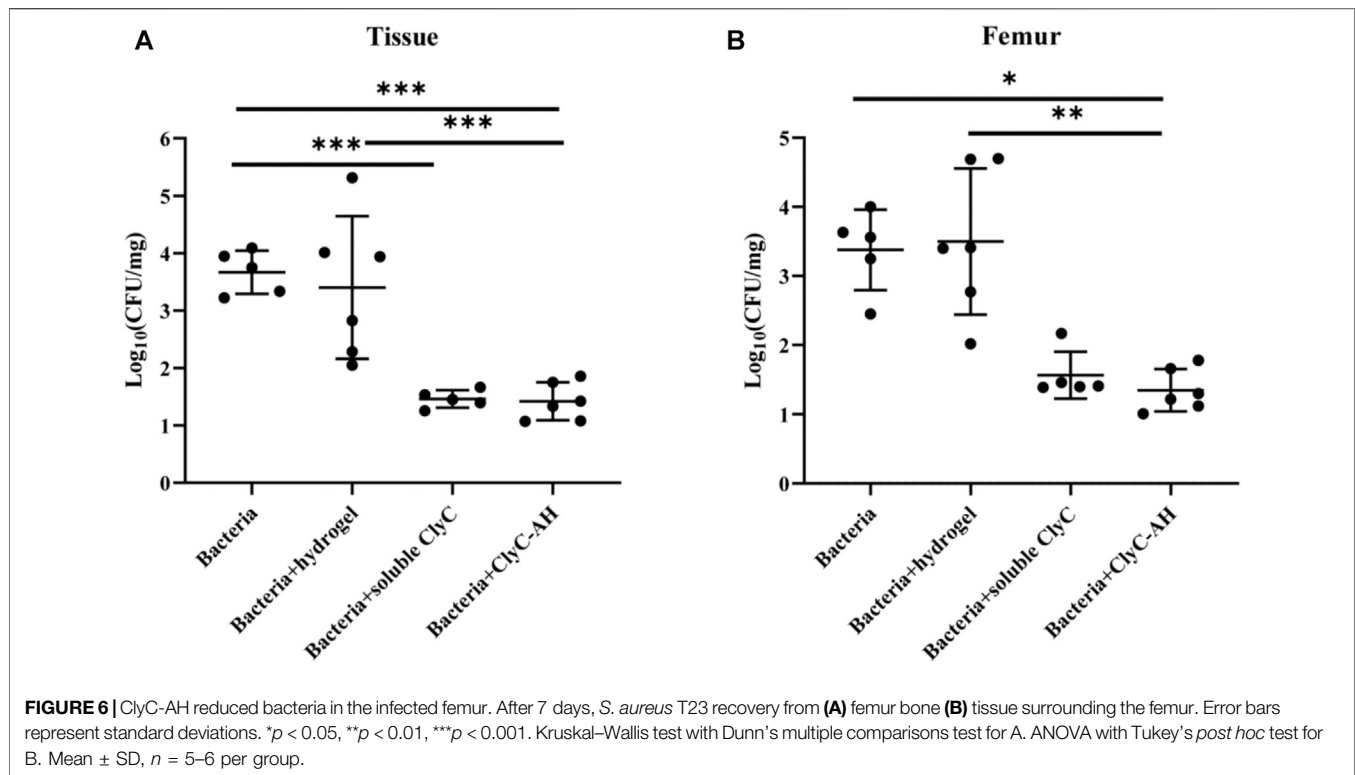
groups had high number of bacteria in the femur and surrounding tissue (**Figure 6**), indicating alginate hydrogels alone had no effect against *S. aureus*. The number of bacteria of the soluble ClyC group was significantly reduced in the surrounding tissue (**Figure 6B**). For the ClyC-AH group, the amount of bacteria was significantly reduced compared with the infection-only group or the hydrogel-only group both in the femur and surrounding tissue (**Figure 6**). In summary, these results demonstrated that ClyC-AH decreased *S. aureus* infections, the effect of which was comparable with that of soluble ClyC.

DISCUSSION

Bacterial infections associated with the outbreak of human diseases have developed into multidrug-resistant forms due to

abuse of antibiotics, necessitating the research and development of new antibacterial agents (Otto, 2012). In this study, we loaded ClyC into the alginate hydrogel, which retained ClyC stability and activity while providing sustained release. Recently, some studies on the encapsulation of endolysin have been reported (Hathaway et al., 2015; Bai et al., 2019; Pinto et al., 2021). Chitosan nanoparticles loaded with the Cpl-1 pneumococcal endolysin showed low cytotoxicity to lung epithelial cell lines and reduced bacterial colonization in the lungs of mice (Gondil et al., 2020a). LysRODI encapsulated in pH-sensitive liposomes reduced *S. aureus* counts by 2log units upon incubation at pH 5 (Portilla et al., 2020).

The delivery system enhanced the bioavailability and safety of the encapsulated protein. *In vitro* release studies revealed that ClyC-AH exhibited biphasic release. There was an initial rapid release, followed by slow and sustained release, which



could maintain the effective ClyC concentration and avoid the rapid degradation of ClyC *in vivo*. The CCK-8 assay also showed that the ClyC-AH exhibited low cytotoxicity, indicating biocompatibility of ClyC-AH.

It is important to stably encapsulate and deliver ClyC without any adverse effects on its structure and antibacterial activity. SDS-PAGE analysis determined the structural integrity of ClyC. It could be explained that proteins could be incorporated into alginate hydrogel under relatively mild conditions, which was also reported by Kaur (Kaur et al., 2020). The hydrogel retained the antibacterial activity of ClyC against *S. aureus* long-term *in vitro*. The anti-bacterial activity of ClyC released from ClyC-AH and ClyC solutions against *S. aureus* was not significantly different at most time points, but the lytic speed of ClyC released from ClyC-AH tends to be slightly slower. This result might be explained by the fewer amount of ClyC released from ClyC-AH at the beginning. Sodium alginate solution can be sol-gel converted by crosslinking some divalent cations (i.e., Ca^{2+}). (Lee and Mooney, 2012). Interestingly, ClyC showed improved activity in the presence of calcium, with up to 9 Log_{10} *S. aureus* cell reduction within 20 min in the presence of Ca^{2+} , which could explain the stable ClyC activity in the hydrogel.

There was no evidence of endolysin resistance, which was still a potential problem. Researchers had demonstrated a synergetic approach to bacterial treatment by a combination of endolysin and antibiotics (Daniel et al.,

2010; Letrado et al., 2018). In our previous studies, we showed that penicillin G and ClyC had a synergistic effect, reducing the MIC of ClyC for planktonic and sessile *S. aureus* (Li et al., 2021). It was beneficial to reduce the working concentration of ClyC in the lesion and weaken potential safety hazards. Next, we will further optimize ClyC-AH by combining with antibiotics to broaden the bactericidal spectrum and decrease the working concentration of ClyC.

Furthermore, results of this study demonstrated antibacterial activity of ClyC-AH against *S. aureus in vivo*. In a *S. aureus* infected mouse model, ClyC-AH significantly reduced bacterial burden compared to the hydrogel-only group. In contrast, mice treated with hydrogel showed no differences in bacterial levels in the femur and surrounding tissue compared to the bacteria-infected control group. This demonstrated that ClyC had antibacterial activity against *S. aureus* infection. However, our results presented one-week observation of mouse osteomyelitis model treatment, which was not enough to make a detailed conclusion. There is a need to extend the experimental period to evaluate the effect of ClyC hydrogel on bone healing and local inflammation.

This study demonstrated that ClyC-AH was successfully prepared by crosslinking Ca^{2+} , with no adverse effect on the structure of ClyC improve the anti-bacterial activity of ClyC. The delivery system could improve the biocompatibility, stability, and prolonged half-life of ClyC. Conclusively, ClyC-AH could be a promising candidate for prevention and treatment of infection caused by *S. aureus*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for Animal Research, Wuhan University.

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YuL, QY and FY designed the project. FY, YiL and XW performed the experiments and analyzed the data. FY drafted the initial manuscript. All authors reviewed the manuscript and read the proof.

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