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Original Article

In vitro antibacterial effect of vancomycin hydrogel on methicillin-resistant Staphylococcus aureus

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Abstract: Objective: To evaluate the *in vitro* antibacterial effect of vancomycin hydrogel on methicillin-resistant Staphylococcus aureus (MRSA). Methods: We used polylactide glycolide-polyethylene glycol-polylactide glycolide (PLGA-PEG-PLGA) copolymer as a carrier of vancomycin to prepare vancomycin hydrogel. A vancomycin hydrogel group, a PLGA-PEG-PLGA copolymer group, a phosphate-buffered saline (PBS) negative control group and a vancomycin group were set for comparison. Then, we analyzed the *in vitro* antibacterial effect of each group to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and to evaluate the effect of vancomycin hydrogel on the cell activity of bacterial biofilms. Results: The temperature of the successfully prepared PLGA-PEG-PLGA vancomycin copolymer was slightly lower than normal body temperature. The copolymer reduced both MIC (1 μg/mL) and MBC (2 μg/mL) for MRSA by 1 time. Compared with phosphate buffered saline negative control group and PLGA-PEG-PLGA copolymer group, the MIC vancomycin and vancomycin hydrogel groups showed a reduction of 3 CFU/mL ($P<0.05$) on the inhibitory effect of original colony count (10^6 CFU/mL). Though the antibacterial effect of MIC the vancomycin group was significantly better than the vancomycin hydrogel group in the first 12 h, the antibacterial effects of the two were similar after 12 hours. The effect of 1 MIC vancomycin on the cell activity of MRSA biofilm was higher than that of 1 MIC vancomycin hydrogel ($P<0.05$). Conclusion: Vancomycin hydrogel with a reduced dosage has a similar antibacterial effect to vancomycin. This finding provides a reference for the development of novel sustained-release vancomycin formulations in future treatment of MRSA.

Keywords: Vancomycin, hydrogel, drug-resistant Staphylococcus aureus, antibacterial effect

Introduction

Staphylococcus aureus can be classified into methicillin-sensitive Staphylococcus aureus and methicillin-resistant Staphylococcus aureus (MRSA) [1]. In recent years, the detection rate of MRSA in China is relatively stable but still at a high level [2, 3]. The treatment for MRSA has become a hot research topic in the medical and pharmaceutical fields. Because of antibiotic overuse, most MRSAs detected in clinical practice are multi-drug resistant bacteria, which are resistant to the antibacterial drugs on the current market to a certain extent [4]. Vancomycin is one of the a few drugs that can effectively inhibit MRSA. It infects the D-Ala-D-Ala dipeptide at the end of MRSA mucin from integrating to dividing cells (the whole intracellular process), impacting greatly cell synthesis and thus kills the bacteria [5]. However, it is revealed that its minimum inhibitory concentration (MIC) for MRSA has shown an increasing trend in recent years [6]. This increase is because vancomycin-resistant bacteria can change the D-Ala-D-Ala dipeptide to no longer be a target of vancomycin and then develop drug resistance [7, 8]. In order to effectively inhabit the development of drug-resistant bacteria, high-dose vancomycin is often used in clinical treatment. However, high dose often brings more toxic side effects, and an effective concentration can hardly be maintained at the infected area. In response to this situation, to develop a new type of sustained-release vanco-

mycin with higher bioavailability is of great significance [9, 10]. Thermosensitive hydrogel is a kind of physical gel that changes from liquid to gel at human body temperature. It is injectable, does not require cross-linking agents and can embed proteins, cells, polypeptides and small molecules etc., showing broad application prospects. Therefore, this study analyzed the *in vitro* antibacterial effect of vancomycin hydrogel on MRSA in order to provide a reference for its clinical use.

Materials and methods

Test strains

This study was approved by the medical ethics committee of the Second People's Hospital of Dongying. The MRSA strains (a total of 40) used in this study were isolated by the Department of Infectious Diseases of our hospital from 2019 to 2021. All the isolation processes were in strict accordance with the standards in *National Clinical Laboratory Procedures* [7]. The stains were further identified and confirmed by cefoxitin disc diffusion test. The standard-quality MRSA strains (ATCC29213) used in this study were purchased from Chinese National Institutes for Food and Drug Control.

Strain recovery and activation

The tryptic soy broth solid medium (BD, US) was prepared according to instructions. A sterilized Tip head was used to dip into the MRSA liquid to smear the liquid on the surface of the medium. Then, the medium was put in an incubator (Shanghai Yiheng, China) at a constant temperature of 35°C for resuscitation culture. After the resuscitation, single colony was streaked by the three-line method and placed in the constant temperature incubator again. After activation, the strains were inoculated into 1 mL medium and continuously cultured. The absorbance was measured by a microplate reader (Tecan, Switzerland) at a wavelength of 490 nm. The growth curve of bacteria was plotted until the bacteria grew to the logarithmic phase.

Preparation of vancomycin hydrogel and gel phase diagram

Poly (ethylene glycol) (PEG, from Purac, Netherlands) was used as a macromolecular initiator to initiate ring-opening polymerization of D, L-lactide and glycolide (both from Purac, Netherlands) in order to obtain polylactide glycolide-polyethylene glycol-polylactide glycolide (PLGA-PEG-PLGA) triblock copolymer [8]. For the synthesis, PEG was put into a three-necked flask, which was then vacuumed to remove water and then cooled down. D, L-lactide and glycolide in a ratio of 10:1 were added to the flask at room temperature. The stannous octoate (95%, from Sigma-Aldrich, USA) was used as catalyst. The reaction was continued for 24 h in nitrogen at 230°C. Thereafter, the prepared synthetic was dissolved in 4°C water and heated to 80°C for 3 repeated cycles to purify the polymer. The purified product was lyophilized and excess water was removed to obtain PLGA-PEG-PLGA copolymer. Phosphate-buffered saline (PBS) was used to prepare it into a copolymer solution at 4°C with a mass of 25%. The copolymer solution was added with vancomycin (specification 0.5 g, H20084268, Zhejiang Haizheng Pharmaceutical Co., LTD.) at a ratio of 10 mg/mL to prepare a 25% copolymer solution.

The prepared vancomycin (10 mg/mL) PLGA-PEG-PLGA copolymer gel was configured at 4°C to a concentration of 15%, 20%, and 25% using PH 7.4 PBS buffer. Then, 0.5 mL of the liquid copolymer was put into a test tube with a diameter of 11 mm, which was then put into a water bath at 10°C. The temperature of the water was precisely increased by 1°C every 10 min until 80°C [9]. During the heating process, the test tube was turned upside down at regular intervals. If the copolymer inside did not flow within 30 seconds, it meant that a gel transition was obtained. That is to say, the corresponding temperature of the water bath is the temperature required for the gel transition of the liquid copolymer. The test was repeated 3 times to obtain an average temperature.

In vitro experiment

In vitro drug release experiment: Vancomycin (0.5 g) was dissolved in 10 mL of water for injection and diluted with 100 mL of sodium chloride (0.9%) to prepare vancomycin for injection. The prepared vancomycin hydrogel (0.5 mL) was put into a glass bottle with a diameter of 16 mm for water bath. After the liquid became gel, 3 mL of PBS diluent was added to each bottle and placed in a 37°C thermostat.

The release experiment was performed at a speed of 60 r/min. Then, at preset intervals, 2 mL of diluent was removed, and the sample was added with another 2 mL of PBS diluent. For the vancomycin control group, the prepared control vancomycin injection was placed into a dialysis bag with molecular weight of 8,000-10,000 Da, and the release medium was phosphate buffer (PH 7.4). The dialysis bag was placed into a 37°C constant temperature water bath and agitated at a speed of 100 r/min. At 6 h, 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h and 96 h after treatment, 1 mL of each sample was taken and added with the same amount of release medium. The vancomycin content in the hydrogel and the control was determined by ultraviolet spectrophotometer (Shimadzu Company, Japan) at a wavelength of 280 nm. Then, the standard curve of release was drawn according to the absorbance values.

Determination of in vitro MIC and minimum bactericidal concentration (MBC): A 96-well microplate (Haimen Shenhua, China) was used to determine the inhibitory and bactericidal concentrations. According to the experimental design, the microplate was divided into 4 groups, vancomycin copolymer group, hydrogel group, vancomycin group and PBS negative control group. The initial concentration of vancomycin was set to 8 μg/mL. In other wells, the concentration was diluted degressively in a 2-fold series. Then, MRSA liquid was added to these wells and incubated at a constant temperature for 12 h to observe and evaluate the MIC. The no growth well next to the MIC well was observed by naked eye, and liquid at this concentration was smeared on the plate to observe if there was no growth of the colony under a microscope to determine the MBC.

Inhibitory and bactericidal experiments: MRSA bacterial solution (100 μL) at a concentration of 10^6 CFU/mL was added with vancomycin hydrogel containing vancomycin at MIC or MBC. Then, the MRSA was added to the wells of different groups (vancomycin hydrogel, MIC vancomycin, MBC vancomycin and PBS control) and incubated in a thermostat at 37°C. To observe and record the number of colonies over time, 10 μL of bacterial solution was taken before incubation, and after 2 h, 4 h, 8 h, 12 h and 24 h of incubation, respectively. The solution was smeared on the agar culture plate directly or after dilution and incubated at 37°C for 24 h. The relevant data were processed using Origin 8.0, and the bacterial growth curve was plotted. The experiments were carried out 3 times in parallel to obtain an average value. The drug that reduced the original colony count by 3 CFU/mL was seen as being able to inhibit MRSA [10].

Preparation of MRSA biofilm: The MRSA solution at the logarithmic growth phase ($2*10^5$ CFU/mL) was diluted and dropped into each well of a 96-well plate. Each well contained 50 μL of bacterial solution and an appropriate amount of tryptic soy broth medium. The plate was then cultivated in an incubator at 37°C for 24 h.

Detection of the effect of vancomycin hydrogel on MRSA biofilm: Pre-cooled PBS was used to wash the biofilms for two consecutive times. The microplate was divided into 4 groups as above, and vancomycin hydrogel was dropped into the corresponding wells. The plate was then incubated for 18 h at 37°C. Thereafter, the solution was added with 100 μL methanol solution (10%) for fixation, stained with crystal violet (0.1%) after 10 min and rinsed with distilled water for 5 min. Ethanol (95%, 100 μL) was then added to fully dissolve the sample, and the absorbance value was detected with a microplate reader at a wavelength of 590 nm.

Detection of the effect of vancomycin hydrogel on cell activity of MRSA biofilm: After washing the biofilms with pre-cooled PBS for two consecutive times. They were incubated with vancomycin copolymer, hydrogel, vancomycin and PBS negative control respectively at 37°C for 18 h. Then, 50 μL of XTT assay solution was added to each well and incubated for another 1 h. The absorbance of the samples was measured when the wavelength of the microplate reader was set as 450 nm.

Statistical methods

Software SPSS 23.0 and Origin 8.0 were used for data analysis. Measurement data met the normal distribution and homogeneity of variance were expressed as mean ± standard deviation, and inter-group comparison was performed by ANOVA or repeated measures ANOVA, followed by inter-group comparison by LSD test. Counting data were expressed as case number or rate (n, %) and processed with

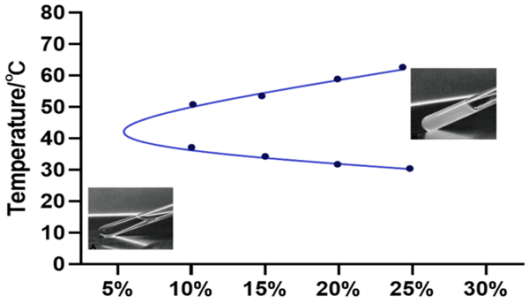

Figure 1. Temperature dependent sol-gel transitions of the copolymer solutions.

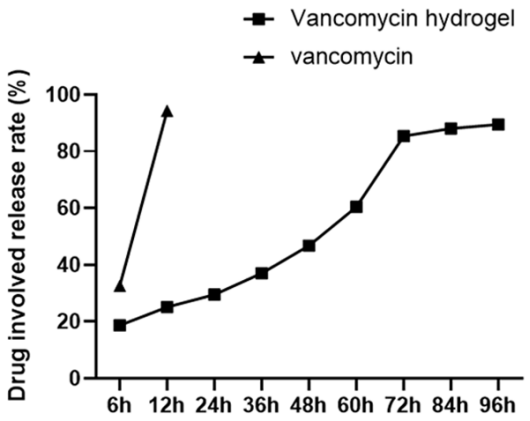

Figure 2. *In vitro* release of vancomycin and vancomycin hydrogel.

the use of Chi-square test. P<0.05 was considered statistically significant.

Results

Preparation of vancomycin PLGA-PEG-PLGA copolymer and sol-gel transition

The PLGA-PEG-PLGA copolymer loaded with vancomycin changed in form with temperature changes *in vitro*. The whole process changed in physical forms of sol-gel-sol (**Figure 1**). As the copolymer concentration increased from 15% to 25%, the gel-form temperature of vancomycin hydrogel decreased, and the temperature increased again when the gel was transitioned to solution. It is suggested that the gel-form temperature of vancomycin hydrogel is slightly lower than normal body temperature. In the course of conventional drug administration, the drug can quickly turn into gel after entering human body, achieving the purpose of local sustained release.

Table 1. MIC and MBC of vancomycin hydrogel for MRSA (μg/mL)

Group	MIC	MBC
PBS negative control group	-	-
Hydrogel group	-	-
Vancomycin group	2	4
Vancomycin hydrogel group	1	2

Note: - indicates that it cannot be determined. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; PBS: phosphate-buffered saline.

In vitro release of vancomycin hydrogel

According to the release standard curve of vancomycin hydrogel in PBS buffer, it can be seen that the release amount of vancomycin hydrogel on the first day was about 30% due to the dispersion effect of the surface. It ensured an effective drug concentration in a short time and exerted the antibacterial effect of vancomycin. After that, the drug was continuously released for about 96 hours, and the cumulative drug release amount reached 89.5%. However, vancomycin released more than 90% after 8-12 h of administration, indicating that the release rate of vancomycin hydrogel *in vitro* was significantly slower than that of vancomycin. See **Figure 2**.

In vitro MIC and MBC of vancomycin hydrogel

Compared with vancomycin group, the MIC (1 μg/mL) and MBC (2 μg/mL) against MRSA were reduced by 1 time in the vancomycin hydrogel group. It is suggested that the degradation of vancomycin hydrogel in the bacterial solution is inhibited to a certain degree. See **Table 1**.

Inhibitory and bactericidal effect of vancomycin hydrogel

Compared with the PBS negative control group, the PLGA-PEG-PLGA copolymer without vancomycin showed no impact on the growth of MRSA, indicating that the PLGA-PEG-PLGA copolymer does not have a bacteriostatic effect. Compared with PBS negative control group and PLGA-PEG-PLGA copolymer group, the MIC vancomycin and vancomycin hydrogel groups showed a reduction of 3 CFU/mL (P<0.05) on the inhibitory effect of original colony count (10^6 CFU/mL). The antibacterial effect of MIC in the vancomycin group was significantly better

Table 2. Sterilization ability of MIC vancomycin hydrogel (lg (CFU/mL))

	PBS	PLGA-PEG-PLGA copolymer	MIC vancomycin	MIC vancomycin hydrogel
0 h	5.01±0.22	5.02±0.19	5.01±0.20	5.03±0.21
4 h	6.21±0.42$^{\wedge\wedge}$	6.15±0.59$^{\wedge\wedge}$	2.42±0.18$^{\#\#,**}$	2.82±0.15$^{\#\#,**,\star}$
8 h	7.06±0.47$^{\wedge\wedge}$	7.02±0.51$^{\wedge\wedge}$	2.24±0.13$^{\#\#,**}$	2.68±0.11$^{\#\#,**,\star}$
12 h	7.75±0.41$^{\wedge\wedge}$	7.71±0.43$^{\wedge\wedge}$	1.83±0.21$^{\#\#,**}$	2.32±0.17$^{\#\#,**,\star}$
16 h	7.96±0.31$^{\wedge\wedge}$	7.95±0.35$^{\wedge\wedge}$	1.33±0.14$^{\#\#,**}$	1.91±0.14$^{\#\#,**}$
20 h	8.13±0.26$^{\wedge\wedge}$	8.15±0.23$^{\wedge\wedge}$	1.05±0.16$^{\#\#,**}$	1.22±0.13$^{\#\#,**}$
24 h	8.59±0.33$^{\wedge\wedge}$	8.64±0.32$^{\wedge\wedge}$	0.58±0.11$^{\#\#,**}$	0.61±0.12$^{\#\#,**}$

Note: Compared with PBS negative control group, $^{\#\#}$P<0.01; compared with PLGA-PEG-PLGA copolymer group, **P<0.01; compared with MIC vancomycin hydrogel group, $^{\wedge\wedge}$P<0.01; compared with MIC vancomycin group, *P<0.05. PLGA-PEG-PLGA: polylactide glycolide-polyethylene glycol-polylactide glycolide; MIC: minimum inhibitory concentration; PBS: phosphate-buffered saline.

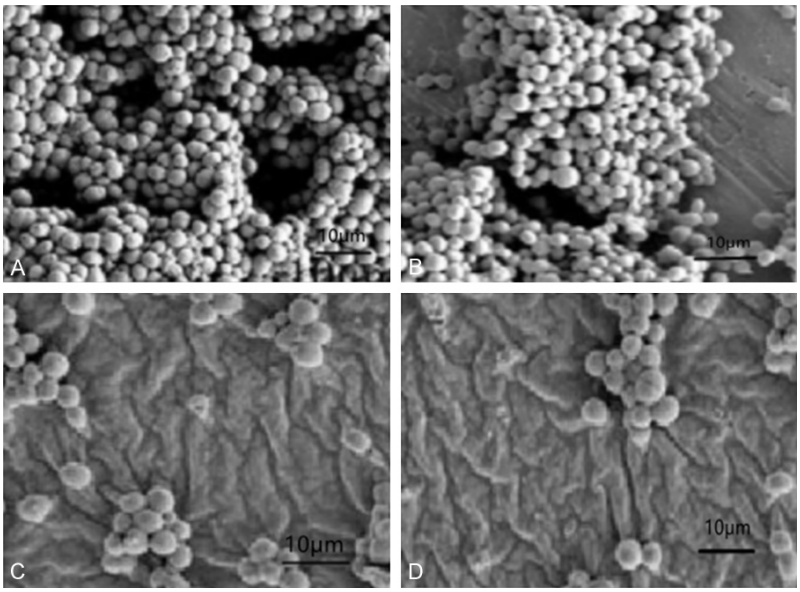

Figure 3. Comparison of colony growth after 24 hours. A: Control group; B: PLGA-PEG-PLGA polymer; C: MIC vancomycin; D: MIC vancomycin hydrogel. PLGA-PEG-PLGA: polylactide glycolide-polyethylene glycol-polylactide glycolide; MIC: minimum inhibitory concentration.

than that of the vancomycin hydrogel group in the first 12 h (P<0.05), but the antibacterial effects of the two groups were basically similar after 12 hours (P>0.05). It can be seen that vancomycin hydrogel, as a local drug release system, has a slower onset bactericidal effect, but it can continuously release vancomycin without adversely affecting the bactericidal effect of vancomycin. See **Table 2** and **Figure 3**.

Impact on the biomass of MRSA biofilm

No inhibitory effect was shown on the biomass of the MRSA biofilm in the PBS negative control group and the PLGA-PEG-PLGA copolymer group. The effect was not significant on the biomass of mature biofilms of MRSA in 1 MIC vancomycin hydrogel and vancomycin groups. When the content of vancomycin increased to 2 MIC, there was still no effect on the biomass of mature biofilms. See **Table 3**.

Effect on cell activity of MRSA biofilm

There was no abnormal effect on the cell activity of MRSA biofilm in the PBS negative control group and the PLGA-PEG-PLGA copolymer group. While 1 MIC vancomycin and 1 MIC vancomycin hydrogel groups showed some lethal effect on cell activity of MRSA biofilm. The lethal effect in the vancomycin group was better than that in the vancomycin hydrogel group (P= 0.030). When the dose reached 2 MIC, the effects of the two groups were basically similar (P>0.05). See **Figure 4**.

Discussion

The hydrogel-antibacterial drug delivery system is based on a local sustained-release drug delivery technology that uses hydrogel as a carrier. As a locally injectable biomaterial, hydrogel can form as solution or gel depending on the temperature, that is, a flowable solution state at room temperature or low temperature, and a gel state at body temperature [11, 12]. As a carrier, hydrogel can coordinate with drugs, peptides, proteins, genes and other substances to achieve local release. In addition, the material is non-toxic and able to control the degradation time in the body according to the different hydrogel materials, so hydrogel does not cause adverse effects to the body and is currently an ideal local sustained-release carrier [13, 14].

Table 3. Impact on the biomass of MRSA biofilms (A 450)

Group	Biofilm biomass
PBS	1.21±0.15▲,★
PLGA-PEG-PLGA copolymer	1.18±0.17▲,★
1 MIC vancomycin	1.19±0.18▲,★
1 MIC vancomycin hydrogel	1.20±0.14▲,★
2 MIC vancomycin	1.16±0.15#,*,△,☆
2 MIC vancomycin hydrogel	1.17±0.16#,*,△,☆

Note: PLGA-PEG-PLGA: polylactide glycolide-polyethylene glycol-polylactide glycolide; MRSA: methicillin-resistant Staphylococcus aureus; MIC: minimum inhibitory concentration; PBS: phosphate-buffered saline. Compared with PBS group, #P<0.05; compared with PLGA-PEG-PLGA polymer group, *P<0.05; compared with 1 MIC vancomycin hydrogel group, △P<0.05; compared with 2 MIC vancomycin hydrogel group, ▲P<0.05; compared with 1 MIC vancomycin group, ☆P<0.05; compared with 2 MIC vancomycin group, ★P<0.05.

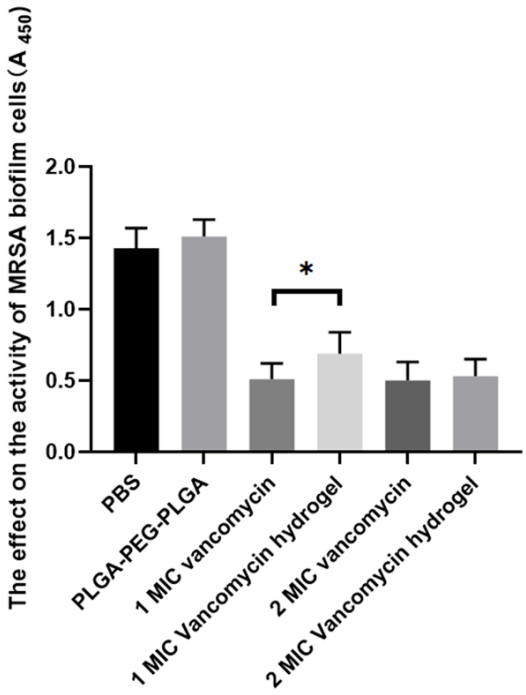

Figure 4. Effect on cell activity of MRSA biofilm. 1 MIC vancomycin hydrogel compared with 1 MIC vancomycin, *P<0.05. PLGA-PEG-PLGA: polylactide glycolide-polyethylene glycol-polylactide glycolide; MRSA: methicillin-resistant Staphylococcus aureus; MIC: minimum inhibitory concentration; PBS: phosphate-buffered saline.

The hydrogel carrier used in this study is PLGA-PEG-PLGA copolymer, which can adjust the degradation time of the copolymer in the body from several weeks to several years according to the ratio of LA/GA and molecular weight [15]. It can be seen from the results that the PLGA-PEG-PLGA copolymer containing vancomycin changed in form with temperature changes *in vitro*. The physical forms changed in sol-gel-sol during the process. As the copolymer concentration increased from 15% to 25%, the gel-form temperature of vancomycin hydrogel decreased, and the temperature increased when the gel was transitioned to solution again. It shows that the prepared PLGA-PEG-PLGA copolymer could change in the gel and solution forms with the temperature change *in vitro*, showing a gel form when it is slightly lower than the body temperature, which ensures a quick transformation to gel after it enters the body. When its temperature increases to 60°C, the gel will transition into solution again, which ensures the hydrogel maintains a gel form in the body.

Wei et al. studied the drug release behavior of PLGA-PEG-PLGA temperature-sensitive hydrogel *in vitro*, and their results indicated that the temperature sensitivity of this PLGA-PEG-PLGA was not affected by the carried drugs [16]. According to the release standard curve of vancomycin hydrogel in PBS buffer, it can be seen that vancomycin-loaded PLGA-PEG-PLGA copolymer can continuously and slowly release the drug in the body. This is mainly because the surface of hydrogel has a certain dispersion effect, which not only ensures an effective drug concentration locally in a short period but also maintains a continues antibacterial effect. Another study dispersed prepared chitosan microspheres in hydrogel, and they injected anti-inflammatory drugs into the knee joint of rabbit model of osteoarthritis. Their results showed that the controlled release of drugs by composite hydrogel could last for over 7 days [17].

Compared with the vancomycin group, the MIC (1 μg/mL) and MBC (2 μg/mL) against MRSA of the vancomycin-loaded PLGA-PEG-PLGA copolymer decreased by 1 time, respectively. Potentially because of the slower degradation rate of hydrogel in the bacterial solution, the degradation of loaded vancomycin is also inhibited to a certain extent in the bacterial liquid. It is indicated that the hydrogel can alleviate the degradation of vancomycin in the bacterial liquid, so that the effect can be continuously released.

A study measured the bacteriostatic effect of vancomycin-loaded fumarate/sodium methacrylate charged copolymer hydrogel on MRSA, and the results showed that the bacteriostatic effect of the polymer was the same as that of vancomycin aqueous solution [18]. Vancomycin hydrogel prepared in this study can reduce the degradation of vancomycin *in vitro* to a certain extent. We also found that the antibacterial effect of MIC in the vancomycin group was significantly better than that in the vancomycin hydrogel group in the first 12 hours ($P<0.05$), but the antibacterial effect of the two groups was basically similar after 12 hours ($P>0.05$). Potentially because when the peak of vancomycin drug passed, the antibacterial effect decreased in MIC the vancomycin group. The effect in the vancomycin hydrogel group was continuous because of the hydrogel. It also further confirmed that vancomycin hydrogel, as a local drug delivery system, has a slower onset of bactericidal effect than the release of vancomycin continuously without adversely affecting the bactericidal effect of vancomycin, which is similar to a related study [19].

PBS negative control and PLGA-PEG-PLGA copolymer alone, PLGA-PEG-PLGA copolymer carrying vancomycin and vancomycin alone all showed no significant effect on the biomass of mature biofilm of MRSA, potentially because vancomycin does not have an obvious bactericidal effect on mature MRSA biofilm. However, both vancomycin hydrogel and vancomycin showed a good bactericidal effect on MRSA biofilm cells. The local administration effectively inhibited the cell activity of MRSA biofilm.

Our results suggested that the PLGA-PEG-PLGA copolymer carrying vancomycin exhibited a slow release and sustained antibacterial effect. The good bactericidal and inhibitory effects on MRSA showed in our study are similar to the results of a related report [20]. During the infection process, Staphylococcus aureus can easily form a biofilm state and become drug-resistant, which brings difficulty for clinical treatment [21]. Through this study, it can be seen that although vancomycin hydrogel showed no obvious inhibitory effect on biofilms, it showed a strong lethal effect on cell activity. By appropriately reducing the vancomycin loading in hydrogel, the purpose of slowing the emergence of vancomycin-resistant MRSA can be achieved to a certain extent.

Although the vancomycin loaded PLGA-PEG-PLGA copolymer shows many advantages, there are still problems to be solved. For instance, study showed that the copolymer could lead to local inflammation during the degradation process in the body, possibly because a certain number of acidic substances would be produced in the degradation process [22]. How to effectively reduce the production of acidic degradation substances requires further research and experiments. Additionally, this is an *in vitro* study and we did not conduct experiments about the *in vivo* effects of the copolymer. We believe that with the continuous advancement of science and technology in the medical field as well as the deepening of research, this new type of biological material will rapidly be developed to help treat human diseases.

In conclusion, the form (sol-gel) of PLGA-PEG-PLGA triblock copolymer carrying vancomycin is temperature-sensitive. This hydrogel has a good antibacterial activity against MRSA, showing stable drug release, which is beneficial to controlling the clinical dose of vancomycin and reducing the occurrence of drug-resistance bacteria. Vancomycin hydrogel has good clinical application prospects.

Disclosure of conflict of interest

None.

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