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A novel injectable chlorhexidine thermosensitive hydrogel for periodontal application: preparation, antibacterial activity and toxicity evaluation

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Abstract The aim of this paper was to evaluate the application potential of CS–HTCC/GP–0.1%Chx thermosensitive hydrogel which was synthesized using chitosan (CS), quaternized CS, and α,β-glycerophosphate (α,β-GP) loading with 0.1% chlorhexidine (Chx) (w/v) for periodontal treatment. An aqueous solution of CS–HTCC/GP–0.1%Chx was transformed into hydrogel at 6 min when the temperature was increased to 37°C. The scan electron microscopy (SEM) image of the gel was a porous, loose and crosslinked network. In vitro, Chx released over 18 h from the CS–HTCC/GP thermosensitive hydrogel in artificial saliva pH 6.8. Release rate could be controlled through adjustment of α,β-GP or Chx concentration. CS–HTCC/GP–0.1%Chx thermosensitive hydrogel exhibited excellent inhibitory activity against primary periodontal pathogens. CS–HTCC/GP–0.1%Chx thermosensitive hydrogel had no acute toxicity; the maximum tolerated dose in rats was 400 mg/ml. All results indicated that CS–HTCC/GP–0.1%Chx thermosensitive hydrogel is a strong candidate as a local drug delivery system for periodontal treatment.

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1 Introduction

Periodontal disease is a chronic infection caused by accumulation of bacterial in dental plaque which produces localized inflammation of the periodontium. And periodontal disease is a possible risk factor for cardiovascular disease (including coronary heart disease, stroke [1] and pre-term low birth weight infants [2].

Dental plaque is the pathogenic source for periodontal disease, and contains $>1 \times 10^{11}$ bacteria/cm^3. Gram-positive and Gram-negative bacteria tend to aggregate and coexist in dental plaque. Numerous studies have shown that the bacterial flora in the periodontal infection area is polymicrobial, i.e., aerobic–anaerobic infections with predominance of anaerobic species. With such a complex and dynamic microbial environment in dental plaque, selection of an effective antibacterial agent is critical, particularly in advanced cases. In recent years, local drug delivery systems against periodontal pathogens have been promoted due to the disadvantages of systemic administration, as well as antibiotic resistance [3, 4].

Chlorhexidine (Chx) is considered a promising antimicrobial agent [5], and possesses a wide spectrum of activity against oral bacteria. In the past 30 years, a Chx local drug delivery system has been widely studied, such as gel [6], chip [7], mouthwash [8] and film [9].

Biodegradable and injectable thermosensitive hydrogels have attracted considerable attention in the past decade [10–14]. They can respond to temperature change: they remain liquid for a long time at room temperature, and turn into a gel if the temperature is increased to 37°C (normal body temperature) while maintaining an effective concentration by a sustained release of antibiotics. Due to these advantages, thermosensitive hydrogels are one of the most convenient and reliable methods to prevent periodontal disease [15].

Chitosan (CS) is a well-known natural parvus polysaccharide. It is a promising biomaterial in the pharmaceutical and medical fields due to its many unique features: biocompatibility; biodegradability; bioactivity; antibacterial activity; and formation of gels and films [16]. Quaternized chitosan *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) is derived from the reaction of CS and glycidyltrimethylammonium chloride (GTMAC) (Fig. 1). This reaction introduced quaternary amino groups into the CS chain to render it soluble in water. Antibacterial and antifungal activity was strengthened, and bioadhesive properties and permeation enhancing effects improved [17, 18].

In this contribution, an injectable thermosensitive hydrogel was synthesized using chitosan (CS), quaternized CS, and α,β-glycerophosphate (α,β-GP) loading with 0.1% chlorhexidine (Chx) (w/v) (CS–HTCC/GP–0.1%Chx) used for periodontal treatment. The in vitro release profile of Chx from CS–HTCC/GP thermosensitive hydrogel was investigated. Antibacterial activity of CS–HTCC/GP–0.1%Chx thermosensitive hydrogel to representative pathogens in the oral cavity—*Porphyromonas gingivalis* (*P. gingivalis*), *Prevotella intermedia* (*P. intermedia*) and *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*)—was determined through determination of minimum inhibitory concentration (MIC) and measurement of the inhibitory zone, which are recommended by CLSI (Clinical and Laboratory Standard Institute) [19]. An in vitro acute toxicity test in rats was done to identify the safety of CS–HTCC/GP–0.1%Chx thermosensitive hydrogel for further clinical application.

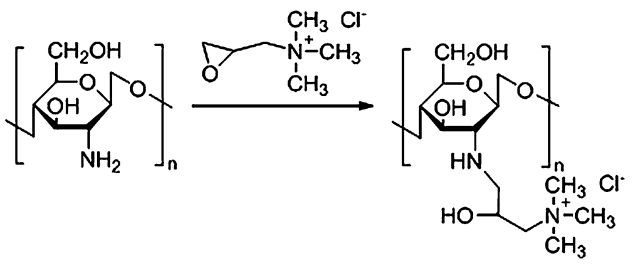

Fig. 1 The chemical structure of CS and HTCC

2 Experimental

2.1 Materials

CS was prepared in our laboratory [20] with 1080 kDa (molecular weight) and 90% (deacetylation degree). GTMAC was obtained from Dongying Guofeng Fine Chemical Company, Limited (Shandong, China). α,β-GP was provided by Kaiyuan Pharmaceutical & Chemical Company, Limited (Shanxi, China). Chlorhexidine was kindly donated by Xi'an Bodyguard Pharmaceutical Company, Limited (Shanxi, China). *P. gingivalis ATCC33277, P. intermedia ATCC 25611* and *A. actinomycetemcomitans Y4* were provided by Beijing Dental Institution, Affiliated Hospital of Capital Medical University (Beijing China). All other chemicals were of analytical grade.

2.2 Synthesis of CS–HTCC/GP thermosensitive hydrogel

HTCC was synthesized by the reaction of CS and GTMAC [21]. Briefly, 6 g CS was mixed and dispersed in 225 ml dimethylcarbinol. The reaction was carried out by stirring at 80–90°C for 1 h. GTMAC was dissolved in deionized water (30% w/v) to form a solution. The GTMAC solution was added to CS suspension slowly. The molar ratio of GTMAC to amino groups of chitosan was 4–1. After 4 h of reaction at 80°C, precipitations were filtered with filter-papers. Then the product was poured into 50 ml alcohol and washed for five times. HTCC was obtained by drying at 80°C for 48 h.

According to the preliminary study [21], the optimum CS–HTCC/GP thermosensitive formulation is (CS + HTCC) (2% w/v), CS/HTCC (5/1 w/w) and α,β-GP 8.33%. Therefore, CS–HTCC/α,β-GP thermosensitive hydrogel was prepared through the following steps. First, 0.15 g CS and 0.03 g HTCC (2% w/v) were mixed and progressively added to 7 ml 0.1 M aqueous lactic acid (LA) solution at room temperature with mechanical stirring until complete dissolution, while adding 2 ml H_2O to dilute the solution. Second, α,β-GP aqueous solution (50% w/v) was prepared in deionized water. Both solutions were chilled in an ice bath for 15 min. Third, α,β-GP aqueous solution (50% w/v) was added drop-by-drop to CS solution under stirring. The final concentration of α,β-GP was 8.33%. The solution obtained was stirred for 20 min. The formulation containing Chx was prepared by pouring the sterilized drug powder to aqueous LA solution before mixing with α,β-GP solution as described above.

CS/GP thermosensitive hydrogel was prepared by progressively adding 0.18 g CS to 7 ml 0.1 M aqueous LA solution. The other steps were identical to those for preparation of CS–HTCC/GP thermosensitive hydrogel as described above.

2.3 Characterization of thermosensitivity

Thermosensitivity of CS–HTCC/GP–0.1%Chx thermosensitive hydrogel at 37°C was determined by the test-tube inverting method [22].

2.4 Morphological studies

Samples of CS–HTCC/GP–0.1%Chx solution and CS–HTCC/GP–0.1%Chx thermosensitive hydrogels were observed after being frozen in liquid nitrogen and lyophilized for 48 h. Samples were then coated with platinum by an ion sputter gold under vacuum. The sample surface was investigated using a scanning electron microscope (KYKY2800B, KYKY Technology Development Limited, Beijing, China).

2.5 In vitro drug release study

Samples (250 mg) with or without Chx were placed in dialysis membranes (New Brunswick, New Jersey, USA) with a molecular weight cut-off of 8000–10,000 to determine the cumulative release rate of the CS–HTCC/GP thermosensitive hydrogel. Dialysis membranes were placed in 100 ml artificial saliva buffer as a simulation of normal gingival crevicular fluid (GCF) and tied at the top to ensure sample retention. The volume of dissolution medium used was dependent on the stage of the dissolution test. Erlenmeyer flasks were placed in a water bath at 37 ± 0.5°C. Buffer solutions were shaken continuously at 100 rpm in a vibrating incubator. At certain intervals, 4 ml buffer was removed and replaced by an equal volume of fresh buffer to maintain a constant volume. Samples were assayed with a UV spectrophotometer (Ultrospec2100 pro, Amershan Biosciences, USA) at 258 nm.

2.6 Antibacterial activity

2.6.1 Bacterial strains and growth conditions

Three representative Gram-negative periodontal pathogens were used: *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans.* Culture medium was prepared by mixing tryptone (1.5%), yeast extract (0.5%), soy peptone (0.55), L-cystine (0.04%), agar (2%) and sodium chloride (NaCl) (0.5%). Before the medium was transferred into sterilized petri dishes, hemin (5 g/ml), vitamin K1 (1%) and defibrinated goat blood (5%) were added to culture at about 50°C. Susceptibility testing of anaerobes was carried according to CLSI/NCCLS [19]. Blood plates with strains were incubated in an anaerobic chamber (Thermo Life Science, USA) under an atmosphere of 80% N_2, 10% H_2 and 10% CO_2 with disoxidation palladium for 72 h. A few singular colonies of the organism were picked from an overnight culture blood agar plate and diluted into sterile physiological saline. The suspension was adjusted spectrophotometrically at 800 nm (optical density$_{800}$) to match the turbidity of 1.5×10^8 CFU ml^{-1} (equivalent to 0.5 McFarland standards) and used further for testing of antibacterial activity.

2.6.2 Test of MIC

The antibacterial agents tested for MIC were LA (0.1 M) solution of Chx, CS, CS–HTCC ($W_{CS}/W_{HTCC} = 5/1$) and CS–HTCC–0.1%Chx ($W_{CS}/W_{HTCC} = 5/1$). The effective concentration of CS was from 5 to 0.00122 mg/ml, and that of Chx was from 2560 to 1.25 μg/ml through a twofold dilution technique. Serial concentration of each sample was mixed in the anaerobic medium mentioned above with 1 ml standard sample mixing with 9 ml anaerobes medium. After the plates solidified at 37°C for 20 min, 100 μl 0.5 McFarland standard organism suspension was inoculated on the surface of blood agar medium plate using 100 μl-Tip. Blood plates were incubated in an anaerobic chamber (Thermo Life Science) with an atmosphere of 80% N_2, 10% H_2 and 10% CO_2 with disoxidation palladium for 72 h.

Control tests were run simultaneously. Each assessment was carried out at three times to ensure reproducibility of results. Two blank controls without antibacterial agent were set up. The MIC was defined as the lowest concentration of the tested sample at which the microorganism colonies were not visible to the naked eye.

2.6.3 Determination of inhibitory zone diameters

The antimicrobial agents were synthesized CS–HTCC/GP–0.1%Chx thermosensitive hydrogel, CS/GP–0.1%Chx thermosensitive hydrogel, and 0.1% Chx solution (0.1 M LA). To determine the inhibitory zone, seeding was done using sterile swabs that were brushed across the agar surfaces in two directions. The 150 μl 0.5 McFarland standard of bacterial suspensions was spread throughout the agar plate. Sterilized stainless-steel tubes of $8.0 \times 1.0 \times 10$ mm (inner diameter, 6 mm) were added to the surfaces of the media, and filled with 100 μl of sterilized antimicrobial solution. Blood plates were incubated in an anaerobic chamber (Thermo Life Science) under an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. After the plates were put into the chamber, the solutions of CS–HTCC/GP and CS/GP changed to the gel after a few minutes. Zones of inhibition of microbial growth around the cylinder containing the tested substances were measured and recorded

after the incubation period. The inhibitory zone was the shortest distance (mm) between the outer margin of the cylinder and the initial point of microbial growth. Six replicates were made for each microorganism. Each assessment was done three times to ensure reproducibility of results.

2.7 Acute toxicity study

Kunming strain mice used in this study were conducted according to Animal Ethical Committee of Medical College, Qingdao University (Qingdao, China), and adhered to the policies and principles established by the Animal Welfare Act and the recommendations in the Guide for Care and Use of Laboratory Animals.

Kunming strain mice (age, 4 weeks) weighing 20–24 g were purchased from Qingdao Experimental Animal Center (Shandong, China: SCXK (lu) 20030010). They were fasted overnight before dosing. A primary experiment revealed that the CS–HTCC/GP–0.1%Chx hydrogel exhibited relatively low toxicity, and LD_{50} could not be determined. Maximal tolerance dose (MTD) was therefore measured using CS–HTCC/GP–0.1%Chx solution.

Twenty mice (10 males and 10 females) were administered CS–HTCC/GP–0.1%Chx solution by stomach gavage at a maximal volume of 0.4 ml/10 g per gavage. Water was provided ad libitum. Daily clinical assessments (intake of food and water, behavior, activity, body weight, and death or killing due to moribundity) were conducted for seven days after dose administration. The MTD was determined.

2.8 Statistical analysis

Statistical data were analyzed using SPSS 13.0 and differences were considered to be significant at a level of $P < 0.05$, using one-way test.

3 Results and discussion

3.1 Characterization of CS–HTCC/GP–0.1%Chx thermosensitive hydrogel

The sol-to-gel transition of CS–HTCC/GP–0.1%Chx took up to 6 min when the temperature was increased to 37°C according to test tube inverting method (Fig. 2). The sol–gel transition time was markedly shorter than that reported for CS/GP thermosensitive hydrogel [23] and HTCC–PEG/GP thermosensitive hydrogel [24].

The substructures of CS–HTCC/GP–0.1%Chx solution and thermosensitive hydrogel are shown in Fig. 3a and b, respectively. After the CS–HTCC/GP–0.1%Chx solution turned into hydrogel, regular holes and a porous structure formed into a crosslinked network (Fig. 3b). The morphological character of the hydrogel made it favorable for water and small molecules to move freely in the network.

3.2 Drug release behavior in vitro

The periodontal pocket provides a natural reservoir that is readily accessible for insertion of a delivery device.

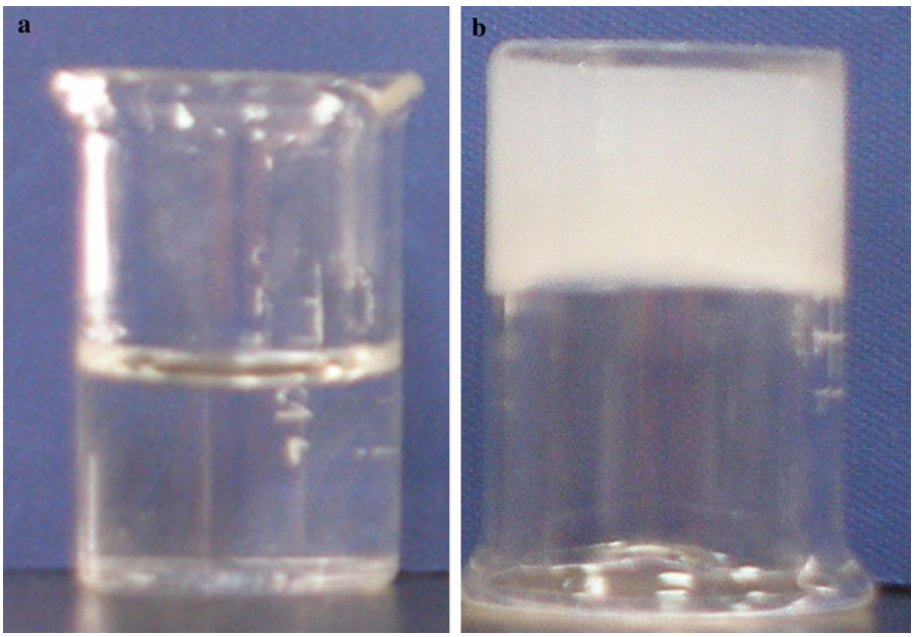

Fig. 2 **a** CS–HTCC/GP–0.1%Chx solution. **b** Formed CS–HTCC/GP–0.1%Chx hydrogel at 37°C

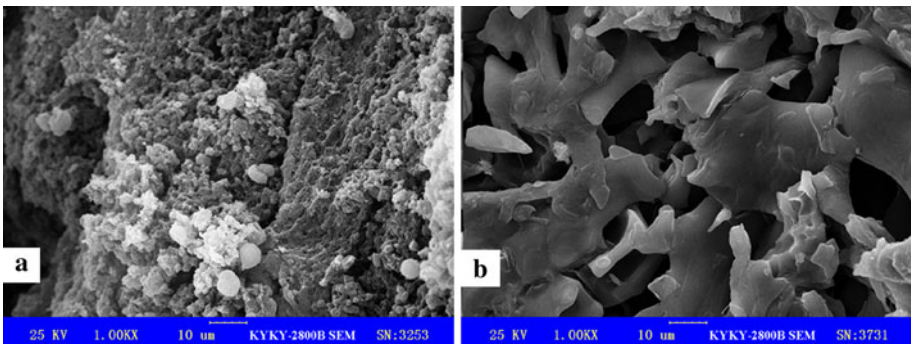

Fig. 3 SEM photographs of CS–HTCC/GP–0.1%Chx solution (**a**) and thermosensitive hydrogel (**b**)

The GCF provides a leaching medium for the release of a drug from the dosage form and for its distribution throughout the pocket. These features, together with the fact that the periodontal diseases are localized to the immediate environment of the pocket, make the periodontal pocket a natural site for treatment with local delivery systems. The speed of drug release is very important for evaluating a new drug delivery system. Various hydrogels for the delivery of tetracycline (2.5%), metronidazole (25%), metronidazole benzoate (40%), as well as a combination of tetracycline (2.5%) and metronidazole benzoate (40%) have been tested and satisfactory results achieved. The gels comprised cellulose derivatives such as hydroxypropylmethyl cellulose [25] and hydroxyethyl cellulose [26–28] and did not appear to have sustained release properties. Despite the rapid release of drug and poor retention of these gels, positive clinical results for moderate-to-deep periodontitis were obtained [29].

The release curves of Chx from thermosensitive hydrogel are shown in Fig. 4a–c. The release profile of Chx from CS–HTCC/GP and the CS/GP hydrogel in artificial saliva buffer pH 6.8 is illustrated in Fig. 4a. Release tendency was similar in CS–HTCC/GP and CS/GP hydrogel, but the cumulative release rate of Chx was slower in the CS–HTCC/GP system than in the CS/GP system using the same medium. Values of 68 and 85% of Chx were released from CS–HTCC/GP and CS/GP hydrogel at 18 h, respectively.

Figure 4b shows the Chx release profiles from hydrogels with different concentrations of GP. Drug release rate was much slower with increasing concentration of GP. GP had an essential role, influencing hydrogel formation by ionic interaction with CS and HTCC. The reaction strengthened the crosslinked network and retarded drug release. With a higher amount of GP added, a more compact hydrogel structure was obtained, and much slower drug release profile observed.

The relationship between Chx concentration in the hydrogel and release ratio is shown in Fig. 4c. The formulation with 0.2% concentration of Chx released faster than that of 0.1% concentration.

In present investigation, the in vitro chlorhexidine (Chx) released over 18 h from the CS–HTCC/GP thermosensitive hydrogel in artificial saliva pH 6.8. The release of Chx was more effectively retarded than that of CS/GP thermosensitive hydrogel. A high concentration of Chx released faster than a low concentration. This result is similar with the study by Senel et al. [9].

According to the results of the release profile, the final concentration of GP as well as drug concentration will alter drug release rates. It is therefore possible to control drug release rates to a relative desired value. Drug release may be by a mixture of diffusion- and degradation-controlled mechanisms. They may be strongly influenced by drug type, polymer chemistry, water sorption and degradation. Though Chx was released from CS–HTCC/GP thermosensitive hydrogel at a relatively higher level and with fast release of drug, it in turn provided sufficient concentrations of drug to be effective at the special site.

3.3 Antibacterial activity

MIC was quantified for all standard bacterial strains selected (Table 1). *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans* were all susceptible to 0.1% Chx with a MIC of 80–160 μg/ml. MICs decreased significantly when Chx associated with CS, especially associated with the compound of CS and HTCC ($5/1W_{CS}/W_{HTCC}$). For example, the MIC of 0.1% Chx solution against *P. gingivalis* was 160 μg/ml. When it associated with CS CS–HTCC ($W_{CS}/W_{HTCC} = 5/1$), the MICs of Chx decreased significantly to 80 and 20 μg/ml, respectively (Table 1). Based on the above results, CS–HTCC–0.1%CHX ($W_{CS}/W_{HTCC} = 5/1$) exhibited a superior antibacterial effect than CS–0.1%Chx and 0.1% Chx.

Inhibitory zones diameters are depicted in Fig. 5. An optical micrograph of an agar plate inoculated with microbials produced by antibacterial samples round the steel tube after one day is shown in Fig. 6. Zones of CS–HTCC/GP–0.1%Chx and CS/GP–0.1%Chx thermosensitive hydrogel continued to increase rapidly in size

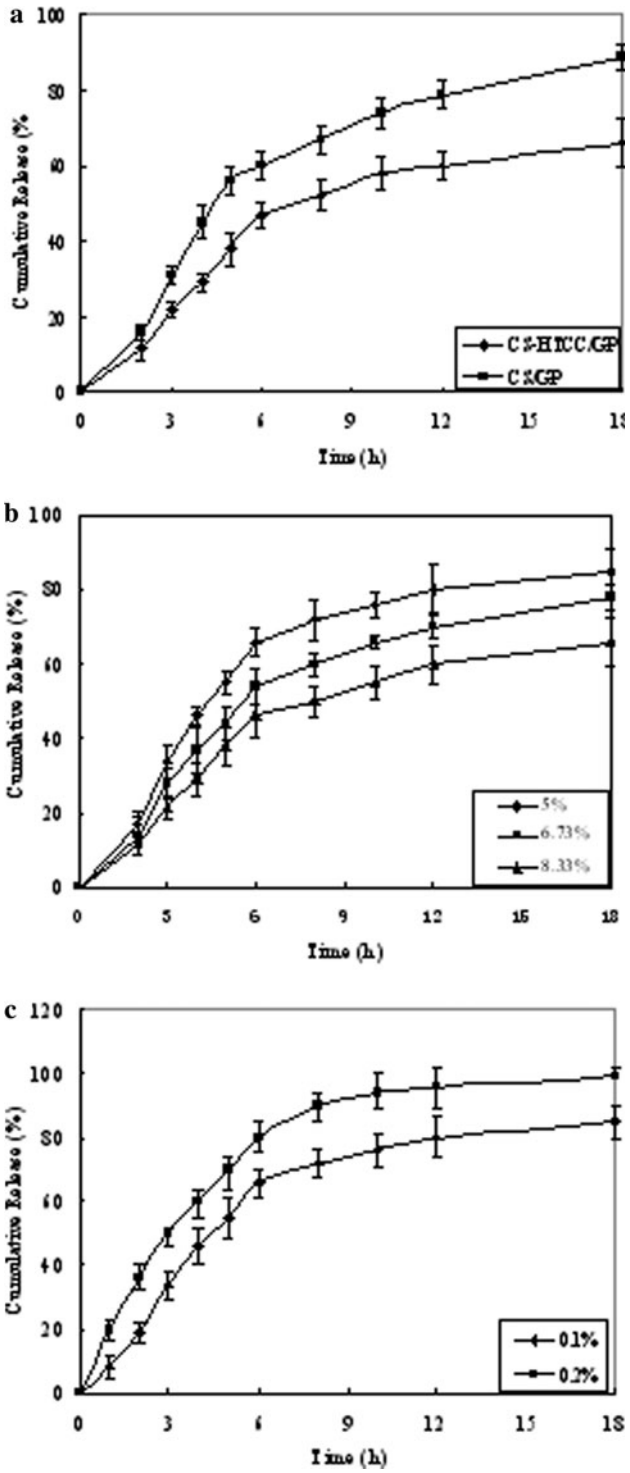

Fig. 4 **a** 0.1% Chx release profiles from CS–HTCC/GP thermosensitive hydrogel and CS/GP thermosensitive hydrogel in artificial saliva pH 6.8 ($n = 3$). **b** 0.1% Chx release profiles from CS–HTCC/GP thermosensitive hydrogel prepared with different concentration of α,β-GP ($n = 3$). **c** Chx release profiles from CS–HTCC/GP thermosensitive hydrogel with different concentration of Chx ($n = 3$)

throughout the duration of this study and the zone of inhibition appeared to reach a plateau after one day. The zone of inhibition obtained with CS–HTCC/GP–0.1%Chx exhibited the strongest inhibition against *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans*, with the largest zone than that of CS/GP–0.1%Chx thermosensitive hydrogel and 0.1% Chx solution alone. The order of antibacterial ability was CS–HTCC/GP–0.1%Chx thermosensitive hydrogel > CS/GP–0.1%Chx thermosensitive hydrogel > 0.1% Chx solution (LA), which was in accordance with measurement of inhibitory zone.

3.4 Acute toxicity study

The acute-toxicity study of CS–HTCC/GP–0.1%Chx thermosensitive hydrogel was conducted with rats following single intragastric administration. In the following seven days after administration, no paradoxical reaction (e.g., toxic effects, body weight loss, inactivity and ingestion decrease) was observed in any group. No death was observed. Based on acute-toxicity data, the MTD was calculated to be 400 mg/kg. This dose is >285 times than that of the clinical daily dose for a 70 kg human (in clinic, Chx concentration in the thermosensitive hydrogel was 0.1%). Data from the in vivo acute toxicity study were valuable for the clinical test, and indicated that CS–HTCC/GP–0.1%Chx was safe.

4 Discussion

P. gingivalis, P. intermedia and *A. actinomycetemcomitans* are the main periodontal pathogens. Anti-periodontal flora studies have shown that CS–HTCC/GP–0.1%Chx is released in an active form and that the gel is suitable for the sustained release of Chx. Chx is an effective and the most widely used anti-plaque agent in antiseptic oral products. CS and its derivates are generally regarded to be antibacterial agents. In our investigation, combination of CS with Chx showed higher activity when compared with Chx or CS alone. CS–HTCC/GP–0.1%Chx thermosensitive hydrogel exhibited the strongest inhibition with the largest inhibitory zone. Combination of CS and Chx can reduce Chx dose and its unwanted side effects.

Staining of teeth and oral tissues [30, 31] is the main drawback of Chx. This may be an aesthetic concern to the patient. This can be avoided in gel form because it is injected locally in the subgingival region.

Concentrations of antibiotic and antibacterial agents generally need to be 100-fold greater to be effective against

Table 1 MIC of antibacterial samples against *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans* (μg/ml)

Antibacterial samples	MIC ($n = 3$)		
	P. gingivalis	*P. intermedia*	*A. actinomycetemcomitans*
0.1% Chx	80	80	80
CS/GP–0.1%Chx solution[a]	31.25 (40)	31.25 (40)	31.25 (40)
CS–HTCC/GP–0.1%Chx solution[a] (5/1 W_{CS}/W_{HTCC})	15 (20)	15 (20)	15 (20)

[a] The value in the bracket represents Chx concentration, and outside the bracket represents the concentration of CS or CS–HTCC

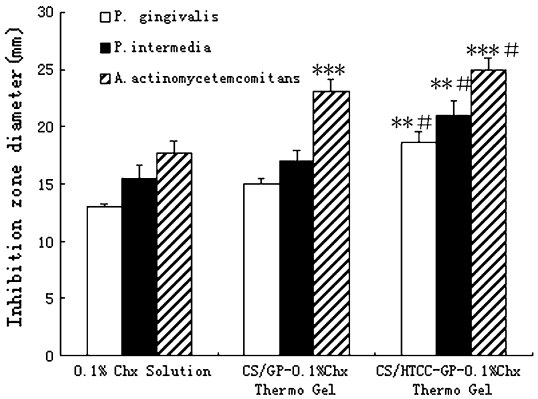

Fig. 5 Average (in mm) zone of inhibitory produced by 0.1% Chx solution (0.1 M lactic acid), CS/GP–0.1%Chx thermosensitive hydrogel and CS–HTCC/GP–0.1%Chx thermosensitive hydrogel against *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans* ($n = 3$). ** $P < 0.01$, *** $P < 0.001$: significant difference compared with 0.1%Chx solution. # $P < 0.05$: significant difference compared with CS–GP–0.1% thermogel

Fig. 6 Optical micrograph of an agar plate inoculated with *P. gingivalis* around steel tube produced by CS–HTCC/GP–0.1%Chx thermosensitive hydrogel after 1 day. The *arrows* point to the zone of inhibition

bacteria within biofilms [32–34]. Assessment of the antibacterial agents that are effective against bacteria within biofilms is required.

CS–HTCC/GP–0.1%Chx thermosensitive hydrogel appears to have all the pre-requisites for clinical evaluation. The aqueous solution of the hydrogel was administered more easily and adapted to the anatomical complexities of periodontal pockets or the pocket size. Hydrogel is bioadhesive to the mucosa in the dental pocket, is biodegradable, and allows controlled release of drug. Bioadhesion study is important physicochemical parameter for in situ forming gels because it prevents the formulation from rapid drainage and hence prolong time. A study on the bioadhension of the gel will be conducted in the following experiments.

Application of the formulation provides an effective drug concentration for an idea period in the periodontal pocket, makes contact with the subgingival flora directly, and suppress or destroys microbial growth. In vivo acute toxicity test of CS–HTCC/GP–0.1%Chx showed no toxicity in rats, with a MTD of 400 mg/ml. Results of in vitro and in vivo studies indicated that CS–HTCC/GP–0.1%Chx thermosensitive hydrogel is an excellent candidate as an intra-pocket drug for periodontal therapy, and CS–HTCC/GP is not only as an ideal vehicle for local delivery of drugs, but also has an active role in the antibacterial process.

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