Controlled release of minocycline in hydroxyapatite/chitosan composite for periodontal bone defect repair

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The aim was to obtain bone repair materials with sustained release of minocycline and evaluate the effect in periodontal bone defect repair. Two complex material, hydroxyapatite/chitosan (HA/CS) and minocycline-hydroxyapatite/chitosan (Mino-HA/CS), were prepared by the co-precipitation method. The physical and chemical property, cytotoxicity, release of minocycline and the bacteriostasis examination of the materials were evaluated, they were applied to the rabbit model of mandible bone defect to evaluate their effects on the regeneration of periodontal bone defect. After minocycline was added to HA/CS, the setting time of the material was prolonged, the compressive strength was reduced and the pore size and porosity were increased significantly. The pH value did not change obviously and stayed in the neutral range. Mino-HA/CS could promote the growth of osteoblasts effectively compared with control medium. In vivo, Mino-HA/CS material showed better effect of promoting periodontal bone formation.

Keywords: Controlled release, Minocycline, Hydroxyapatite/chitosan composite, Bone defect repair

INTRODUCTION

In recent years, biosynthetic materials for bone defect repair have received more and more attention. In the oral environment, the wound after bone defect repair may be difficult to heal if the local bacterial factors are not properly controlled. In such cases, traditional systemic use of antibiotics is not only ineffective, but may lead to multiple side effects and accumulation of drug toxicity. Therefore, there is an urgent need for a biological material that has bacteriostatic function. Not only should it release an effective concentration of antibiotics for extended periods of time, but it should also have good biocompatibility which can promote tissue growth.

Hydroxyapatite, a main component of vertebrate bones and teeth, has good biocompatibility and can be used alone or as a scaffold for tissue engineering to repair bone defects1-20. Chitosan, the deacetylation product of chitin, is an alkaline polysaccharide. It is nontoxic and non-irritating to human tissue, has good biocompatibility, biodegradability, antibacterial, anti-tumor activity, and adhesion properties, and has analgesic and hemostatic effects21-40.

Compounding hydroxyapatite with chitosan as bone grafting materials, the compressive strength, degradability and injectability are improved. Hydroxyapatite/chitosan (HA/CS) implants can promote the adhesion of proteins and bone tissue cells on the surface of the implanted material. It has the potential to induce bone marrow mesenchymal stem cells to differentiate into osteoblasts and promote their proliferation, which is beneficial to the formation and remodeling of new bone41. In addition, HA/CS has antibacterial properties42. In recent studies, HA/CS were reported as drug carriers for Chinese medicine, protein drugs, and antibiotics to achieve sustained release43-48. Minocycline (C23H27N3O7), commonly used in the treatment of periodontitis, is a second generation semi-synthetic tetracycline broad-spectrum antibiotic. It is active against aerobic, anaerobic, Gram-positive and Gram-negative bacteria. It can enhance bone formation, decrease connective tissue breakdown, and reduce bone resorption49-51. It has been widely used in the clinical treatment of gingivitis periodontitis. However, the long-term antibacterial effect of minocycline in bone formation was inhibited due to its rapid metabolism, a method needs to be implemented to achieve its controlled release.

In the application of antibacterial artificial bone in prosthodontics, in order to ensure the porosity and compressive strength, it was difficult to achieve the controlled release of drugs in the previously reported antibacterial artificial bone. Therefore, we tried to use HA/CS as the carrier of minocycline, and improved the synthesis process in order to improve the defect of poor controlled release of antibacterial materials. The properties, drug release, biocompatibility, bacteriostasis and effect on periodontal bone repair were evaluated comprehensively. The results provide an experimental
basis for its application in clinical periodontal bone defect repair.

**MATERIALS AND METHODS**

**Reagents**

Major reagents used in the experiment including the following: Chitosan (Golden Shell, Shanghai, China); Citric Acid, CaCl₂, NaH₂PO₄, CH₃COOH, Ca(OH)₂, H₃PO₄, Anhydrous Ethanol (SINOPHARM, Shanghai, China); poloxamer, CaCO₃, NaHCO₃, minocycline (Sangon Biotech, Shanghai, China); Porphyromonas gingivalis ATCC33277 (The National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China); Staphylococcus aureus ATCC25923 (The National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China); MC3T3 cells (Shanghai Cell Bank, Shanghai, China); New Zealand rabbits (Second Military Medical University Animal Center, Shanghai, China).

**Composite formulation and paste preparation**

A co-precipitation method was used to prepare nano-HA/CS composite. Ca(OH)₂ suspension was prepared by suspending Ca(OH)₂ in absolute alcohol. A chitosan aqueous solution of 3 wt% was prepared by dissolving the chitosan powder into distilled water containing 2 wt% acetic acid. The solution was then added to a 10 wt% H₃PO₄ solution. The ratio of CS to H₃PO₄ was adjusted to form the final HA/CS ratio of 80/20. The CS/H₃PO₄ solution was slowly added into the Ca(OH)₂ suspension. After stirring and mixing for 12 h at room temperature, the slurry obtained was aged for 24 h. The precipitate was filtered and washed with distilled water, and was then freeze-dried and pulverized by the high-speed centrifugal pulverizer to obtain the composite material powder.

One gram of solid powder (72.55% HA/CS, 17.25% Ca(OH)₂, 3.1% CaCO₃, 3.1% NaHCO₃ and 4% Minocycline) was mixed with 2 mL setting liquid (3.2% Ca, 3.6% CH₃COOH, 1.6% NaH₂PO₄, 0.8% CaCl₂, 0.4% poloxamer, and 0.4% CS) to make the Minocycline-loaded cement paste. Then the paste was loaded into syringe (the weight of each sample is 0.15±0.01 g), set in the MHA hole by the sterile forceps and put in the incubator at 37°C. The composite was punched in the MHA, the composite material was spreaded evenly and the diameter of the inhibition zone was recorded each day as well.

**Bacteriostasis examination**

The Staphylococcus aureus solution was mixed well with Mueller-Hinton agar (MHA). A hole of the same size as the material was punched in the MHA, the composite (the weight of each sample is 0.15±0.01 g, including 4 wt% minocycline or not) was placed on the blood plate by sterile cotton swabs. The composite was placed in a 96-well plate, completely immersed with 150 µL of simulated saliva, and held at 37°C for 28 days. All the dissolution medium was replaced and collected daily, and the release of minocycline was detected by HPLC method.

**Minocycline in vivo release behavior**

The weight of each sample was controlled at “0.15±0.01 g”, including 4 wt% minocycline, and the samples were placed in a 96-well plate, completely immersed with 150 µL of simulated saliva, and held at 37°C for 28 days. All the dissolution medium was replaced and collected daily, and the release of minocycline was detected by HPLC method.

**Defect and repair of rabbit mandible**

Twelve white male New Zealand rabbits, ages ranging from 6–7 months old and weighing 2.6–3.1 kg were used in this study. They were housed in the laboratory animal center at the Chinese Second Military Medical University. All surgical procedures and care followed Second Military Medical University Laboratory Animal Ethics Committee. A total of 24 bone defects were prepared on the bilateral mandibles of rabbits. The bone defects were randomly divided into A: blank group (bone defect was not filled in), B: HA/CS group, and C: Mino-HA/CS group. Postoperatively, penicillin sodium was intramuscularly injected from 100,000 to 200,000 units for 24 to 48 h and injected continuously for three days.
The stitches were removed 10 days after the operation. The rabbits were sacrificed at 8 and 12 weeks postoperatively, and the whole layer of each defect bone tissue and its 10 mm surrounding area were respectively resected and analyzed by HE staining. The histological evaluation was performed by Wodeng Biotechnology Company with blind method. Image analysis software (Image-Pro Plus 6.0) was used for quantitative analysis, and the percentage of new bone area (NBA) in total defect area (TDA) was calculated for each image\(^{14}\). All surgical procedures and care followed Chinese PLA General Hospital Review Board approval (Approval number: SYXX (ShangHai) 2017-0004).

**Statistics**

Student t-test was used for comparisons between two groups. The significance level was set at \( \alpha = 0.05 \) for all statistical tests. All statistical analyses were performed using SPSS 18.0 software package.

### RESULTS

HA/CS and Mino-HA/CS filler material were synthesized respectively and the physical and chemical properties were tested. As shown in Table 1, the initial setting time and the final setting time of HA/CS and Mino-HA/CS were maintained within 10–30 min, which can provide sufficient time for clinical operations. The pH of two-group extraction solution was in the neutral range (pH 6.95–7.32), from the initial release period (day 1) to the stable release period (day 7).

After the addition of minocycline, the compressive strength of Mino-HA/CS was decreased compared by HA/CS (117.6 MPa vs. 242.9 MPa), while the compressive strength of both groups was similar to that of dense bone. Meanwhile the porosity of Mino-HA/CS was increased significantly (54.25% vs. 43.73%), it was also observed in SEM (Fig. 1), which might promote the adhesion and growth of osteoblast.

The cytotoxicity to MC3T3 cells was tested at 48 and 72 h. The results were described as Mean±SD. *: \( p<0.05 \), **: \( p<0.01 \).

### Table 1 Physical and chemical property and cytotoxicity of HA/CS and Mino-HA/CS

| Setting time (s) | Compressive strength (MPa) | pH | Porosity (%) | RGR%  |
|-----------------|---------------------------|----|--------------|-------|
| Initial         | Final                     | Day 1 | Day 7 | 48 h | 72 h |
| HA/CS           | 13.0±0.4                  | 242.9±33.6 | 6.97±0.01 | 43.73±2.82 | 98.1 | 106.6 |
| Mino-HA/CS      | 15.0±1.2*                 | 24.5±2.0* | 117.6±31.1** | 6.95±0.01 | 54.25±1.47** | 105.8 | 116.1 |

The SEM images.

[A] The surface of HA/CS; [B] The cross-sections of HA/CS; [C] The surface of Mino-HA/CS; [D] The cross-sections of Mino-HA/CS. Mino-HA/CS presents a greater number of pores and larger size holes.
72 h respectively, compared with complete medium, the extract of HA/CS and Mino-HA/CS showed no cytotoxicity (cytotoxicity grade \( \leq 1 \)) within 48 h. After 72 h culture, cells grown in the extract of both HA/CS and Mino-HA/CS showed better relative growth rate than control (Fig. 2 and Table 1). SEM also showed that osteoblasts crawled on the surface and in the cross section of Mino-HA/CS and HA/CS, more osteoblasts attached to the surface and pores of Mino-HA/CS (Fig. 3).

HPLC was used to determine the release of minocycline. As shown in Fig. 4A, the daily release of minocycline reached a peak of 3.97 μg on the second day, and then decreased day by day thereafter. After one week, the release rate reached a steady state, which was maintained at 0.5–1 μg/d for 28 days at least.

According to the results of minocycline release, the antibacterial effect of Mino-HA/CS on *Staphylococcus aureus* and *Porphyromonas gingivalis* was determined. Bacterial plates were replaced daily, the Mino-HA/CS showed good antibacterial property against both *Staphylococcus aureus* and *Porphyromonas gingivalis*. The average diameter of the inhibition zone was the largest on the second day, which is consistent with the results of minocycline release. After one week, the diameter of the inhibition zone could still greater than 20 mm, indicating that the Mino-HA/CS has a long-term inhibitory effect on growth of both *Staphylococcus aureus* and *Porphyromonas gingivalis* (Figs. 4B, C).

Bone defects were prepared on the bilateral mandibles of rabbits, the defects were filled with HA/CS, Mino-HA/CS or without materials respectively (Fig. 5). After 8 weeks, early new bone formation was observed by HE staining. Early bone tissue was observed to form at the edge of the wound, HA/CS and the Mino-HA/CS group had significantly less connective tissue than the blank group, which may be more conducive to the crawling of bone tissue toward the center. At 12 weeks, more bone tissue was formed toward the center. Compared with the blank group, more new bone tissue was observed in HA/CS and the Mino-HA/CS group, in
Fig. 4  Release of minocycline and antibacterial effect of Mino-HA/CS.
[A] Daily and cumulative release of minocycline in Mino-HA/CS (n=6); [B] Diameter of antibacterial zone of Mino-HA/CS for *Staphylococcus aureus*; [C] Diameter of antibacterial zone of Mino-HA/CS for *Porphyromonas gingivalis*.

Fig. 5  Surgical treatment of mandible defect.
[A, B] 20 mm incision was made around 0.5 mm above the lower margin of the mandible in all rabbits. A segmental defect (10×5×3 mm) was prepared 2 mm in front of the mental foramen by Kavo K4; [C] The two groups of materials were placed in the prepared defects; [D] The periosteum, muscle layer, and skin were sutured.
Fig. 6 Typical histological images of bone defect sites at 8 and 12 weeks. Early bone tissue was observed to form at the edge of the wound after 8 weeks in group A (blank group) [A1, A2], group B (HA/CS) [B1, B2] and group C (Mino-HA/CS) [C1, C2]. At 12 weeks, more bone tissue was formed toward the center in group A (blank group) [A3, A4], group B (HA/CS) [B3, B4] and group C (Mino-HA/CS) [C3, C4].

Table 2 Rate of new bone formation

| Week | Control (%) | HA/CS (%) | Mino-HA/CS (%) |
|------|-------------|-----------|----------------|
| 8    | 44.6±4.8    | 49.7±1.7  | 65.5±4.4<sup>a</sup><sup>b</sup> |
| 12   | 66.3±2.9    | 74.5±1.7  | 90.6±2.5<sup>a</sup><sup>b</sup> |

Note: The results were described as Mean±SD (n=4).<sup>a</sup> p<0.01 compared with Control;<sup>b</sup> p<0.01 compared with HA/CS

pulverized and sieved to prepare HA/CS granules. The granules were then blended with the solidification liquid and other ingredients. At this point, the solidified solution contained water-soluble chitosan coated on the surface of the HA/CS particles. In this synthetic method, Ca<sup>2+</sup> in hydroxypatite can be combined with the chitosan amine<sup>15</sup>. This competitive wraparound effect may reduce the chelation of minocycline and hydroxyapatite. In addition, mixing the HA/CS particles with minocycline powder and then mixing with the chitosan-containing solidification solution can make part of the minocycline encapsulated by chitosan. This may play a role in reducing the initial burst release of minocycline.

On the other hand, in-situ solidification produces CO<sub>2</sub>, which forms a porous structure to increase pore size and porosity. The porosity increased from 43.68% to 53.99% and the pore size was able to reach 20-200 μm after adding minocycline. The higher porosity and
larger pore size of the bone graft material can facilitate the entry of tissue fluid or blood into its internal network structure, allowing the continuous diffusion of drugs and biologically active molecules through the pore channels to the surrounding tissue\textsuperscript{16,17}.

The pH of the bone graft material is particularly important in vivo. A strong acidity or alkalinity bone grafting material will cause cytotoxicity and affect the tissue growth. In our experiment, the pH value of composite materials both before and after loading tended to be stable after reaching 7.3 on the fourth day. HA/CS and Mino-HA/CS is nontoxic to the MC3T3 cells and has good biocompatibility.

Bone defects in oral cavity are often accompanied by bacterial infection. It has been reported that 85% of subgingival plaque bacteria are inhibited when the concentration of minocycline is 1 μg/mL. The main pathogen of periodontitis, Porphyromonas gingivalis, has an MIC90 of less than 0.1 μg/mL and the MIC90 of Prevotella intermedia is less than 0.39 μg/mL\textsuperscript{18}. The minocycline released during the first month of this study can maintain about 0.5–1 μg/d, which can inhibit most of the periodontal pathogens. The inhibition zone also proved that Mino-HA/CS has a good antibacterial effect against Staphylococcus aureus and Porphyromonas gingivalis.

In the in vivo experiment, we selected the rabbit’s mandible to establish a bone defect model. Although no bacteria were implanted in the bone defect, the experimental group filled with Mino-HA/CS still showed more effective bone formation. In the follow-up test, we will further evaluate the effect of Mino-HA/CS on bone formation in the presence of oral harmful bacteria.

In conclusion, a Mino-HA/CS composite with controlled release for minocycline was obtained in this study. This material can promote cell proliferation and tissue growth while releasing a safe dose of minocycline for a long period of time. It can inhibit Staphylococcus aureus and Porphyromonas gingivalis bacteria effectively. This finding provides a theoretical and experimental basis for the future application of Mino-HA/CS in the repair of periodontal bone defects and regeneration of periodontal tissues.

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